extension of this time limit may be granted under either 37 C.F.R. 1.136 (a) or (b), but the period for response set in the last office action may be extended up to a maximum of six months.

In response, applicants respectfully traverse the Examiner's provisional obviousness-type double patenting rejection. Applicants maintain that the content of claims 44 and 46-56 in the subject application differs from the content of claims 1-20 and 44-52 in copending U.S. Serial Nos. 08/475,784 and 08/477,097. In the subject application, applicants' claims are directed to methods of using a vaccine.

Applicants further point out that for a provisional double-patenting or obviousness-type double patenting rejection, M.P.E.P. §804 requires that the:

> 'provisional' double patenting rejection should continue to be made by the Examiner in each application as long as there are conflicting claims in more than one application unless that 'provisional' double patenting rejection is the only rejection remaining in one of the applications. If the 'provisional' double patenting rejection in one application is the only rejection remaining in that application, the Examiner should then withdraw that rejection and permit the application to issue as a patent, thereby converting the 'provisional' double patenting rejection in the application(s) into a double patenting rejection at the time the one application issues as a patent.

Therefore, applicants maintain that even if the Examiner continues to conclude that the claims of the subject application conflict with the claims of U.S. Serial Nos. 08/475,784 and 08/477,097, the provisional rejection should be withdrawn in view of applicants' arguments which overcome the other rejections of this application under Sections 101, 103 and 112. Thus, the subject application should be allowed to issue.

In view of the foregoing statements, applicants respectfully

request that the Examiner reconsider and withdraw the rejection based on provisional obviousness-type double patenting.

SECOND COMMUNICATION REQUESTING CORRECTED FILING RECEIPT

This Second Communication is filed to request a corrected Filing Receipt in connection with the above-identified application. Applicants previously filed a Communication To Correct Error In Filing Receipt with the U.S. Patent and Trademark Office on November 13, 1995; a copy is attached hereto as **Exhibit A**. Applicants have not received a corrected Filing Receipt in response to the November 13, 1995 Communication

Upon receipt of the official Filing Receipt for the subject application, a copy of which is attached hereto as **Exhibit B**, applicants' undersigned attorney noticed errors.

Applicants hereby again respectfully request that a corrected Filing Receipt be issued. Specifically, after "CONTINUING DATA AS CLAIMED BY APPLICANT-",the following now appears:

THIS APPLN IS A CON OF PCT/US94/00757 01/21/94
AND A CON OF 08/009,628 01/21/93 PAT 5,333,920

A corrected Filing Receipt should read as follows:

--THIS APPLN IS A CON OF PCT/US94/00757 01/21/94
AND A CIP OF 08/009,268 01/22/93 ABN--

Also, after "TITLE", the following now appears:

GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS OS-21

A corrected Filing Receipt should read as follows:

--GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS QS-21--

Applicants contend that the failure to change the Filing Receipt and issue a Corrected Filing Receipt is due to delay by the United States Patent and Trademark Office. As stated above, a Communication To Correct Error In Filing Receipt was filed on November 13, 1995, including a check in amount of $25.00 required under 37 C.F.R. §1.19(h) for the issuance of an additional filing receipt corrected due to applicants' error. Accordingly, applicants again respectfully request that a corrected Filing Receipt be issued.

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

In accordance with their duty of disclosure under 37 C.F.R. §1.56(c) and §1.97(c), applicants would like to direct the Examiner's attention to the following disclosure which is listed on the attached Form PTO-1449 (Exhibit 1) and attached hereto as Exhibits 2-6:

1. Helling, F., et al. (1993) "Increased immunogenicity of GM2 conjugated with KLH and used with adjuvants in patients with melanoma." Proceedings Of The 84th Annual Meeting Of The American 34: 491 (Exhibit 2);

2. Helling, F., et al. (1993) "Construction of immunogenic GD-3 conjugate vaccines." Annals Of The New York Academy Of Sciences 690: 396-397 (Exhibit 3);

3. Helling, F., et al. (1994) "GD3 vaccines for melanoma superior immunogenicity of keyhole limpet hemocyanin conjugate." Cancer Research 54: 197-203 (Exhibit 4);

4. Bystryn, J-C (1990) "Tumor vaccines." Cancer And Metastasis Reviews 9: 81-91 (Exhibit 5); and

5. Ritter, G., et al. (1990) "Induction of antibodies reactive with GM2 ganglioside after immunization with lipopolysaccharides for Camplyobacteria jejuni." Int. J. Cancer 66: 184-190 (Exhibit 6).

The references, enclosed as Exhibits 2-5, were cited in the Supplementary European Search Report issued in connection with European Application No. 94 90 7279.7, filed August 22, 1995, corresponding to PCT International Application No. PCT/US94/00757, filed January 21, 1994. The subject application is a continuation application of PCT/US94/00757. A copy of the Supplementary European Search Report is attached hereto as **Exhibit 7.**

The reference, enclosed as Exhibit 6, was cited by the New Zealand Patent Office during the substantive examination of New Zealand Application No. 261744, filed July 28, 1995, corresponding to PCT International Application No. PCT/US94/00757, filed January 21, 1994. The subject application is a continuation application of PCT/US94/00757.

Applicants maintain that the subject invention is novel and nonobvious over the teachings disclosed in the above-identified publications. Accordingly, applicants maintain that the above-identified publications neither disclose nor suggest the invention claimed in the subject application.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the $230.00 fee under 37 C.F.R. §1.17(p), is deemed necessary in connection with the filing of this

Communication In Response To April 15, 1997 Office Action, Second Communication Requesting A Corrected Filing Receipt and Supplemental Information Disclosure Statement. However, if any other fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

Albert Wai Kit Chan

John P. White
Registration No. 28,678
Albert Wai-Kit Chan
Registration No. 36,479
Attorneys for Applicant(s)
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1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Albert Wai Kit Chan 5/2/97

Albert Wai-Kit Chan Date
Reg. No. 36,479

Attachment of Paper No. 12

Dkt. 43016-D/JPW/AKC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Philip O. Livingston and Friedhelm Helling

Serial No.: 08/477,147

Filed : June 7, 1995

For : GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS QS-21

1185 Ave of the Americas
New York, New York 10036
November 13, 1995

Assistant Commissioner for Patents
Washington, D.C. 20231

MAY 2 2 1997

SIR:

COMMUNICATION TO CORRECT ERROR IN FILING RECEIPT

This Communication is submitted to request correction of an error in the filing receipt issued by the United States Patent and Trademark Office in connection with the above-identified application. Applicants' undersigned attorney received the filing receipt on October 13, 1995. A copy of the filing receipt is attached hereto as Exhibit A.

Applicants hereby respectfully request the following corrections be made:

> The title of the invention given as "GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS OS-21" should be as follows --GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS QS-21--.
>
> The second line in the Continuing Data As Claimed By Applicant given as "WHICH IS A CON OF 08/009,628 01/21/93 PAT 5,333,920" should be as follows --WHICH IS A CIP OF 08/009,268 01/22/93 ABANDONED--.

A fee of TWENTY-FIVE DOLLARS ($25.00) is required according to 37 C.F.R. §1.19(h) for the issuance of an additional filing

receipt corrected due to applicant error. A check for $25.00 is enclosed to cover this fee.

No fee, other than the $25.00 fee for the corrected filing receipt, is deemed necessary in connection with the filing of this Communication. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

Albert Wai Kit Chan

I hereby certify that this paper is being deposited this date with the U.S. Postal Service as first class mail addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Albert Wai-Kit Chan 11/13/95

Albert Wai-Kit Chan Date
Reg. No. 36,479

John P. White
Registration No. 28,678
Albert Wai-Kit Chan
Registration No. 36,479
Attorneys for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

PTC-103X
(Rev. 8-95)

FILING RECEIPT

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Offic
ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washingt n, D.C. 20231

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
08/477,147	06/07/95	1806	$904.00	43016-D/JPW/	26	22	1

JOHN P WHITE
COOPER & DUNHAM
1185 AVENUE OF THE AMERICAS
NEW YORK NY 10036

Receipt is acknowledged of this nonprovisional Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please write to the Application Processing Division's Customer Correction Branch within 10 days of receipt. Please provide a copy of the Filing Receipt with the changes noted thereon.

Applicant(s)

PHILIP O. LIVINGSTON, NEW YORK, NY; FRIEDHELM HELLING, NEW YORK, NY.

CONTINUING DATA AS CLAIMED BY APPLICANT-
THIS APPLN IS A CON OF PCT/US94/00757 01/21/94
WHICH IS A CON OF 08/009,628 01/21/93 PAT 5,333,920

FOREIGN FILING LICENSE GRANTED 10/05/95
TITLE
GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS OS-21

PRELIMINARY CLASS: 424

(see r vers)

Philip O. Livingston and Friedhelm Helling
U.S. Serial No. 08/477,147
Filed: June 7, 1995
Exhibit B

Form PTO-1449

INFORMATION DISCLOSURE CITATION IN AN APPLICATION

(Use several sheets if necessary)

Docket Number (Optional)	Application Number
43016-D/JPW/AKC/JKM	08/477,147
Applicant: Philip O. Livingston and Friedhelm Helling	
Filing Date: June 7, 1995	Group Art Unit: 1806

U. S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

AC	Helling, F., et al. "Increased immunogenicity of GM2 conjugated with KLH and used with adjuvants in patients with melanoma." March 1993 Proceedings Of The 84th Annual Meeting Of The American Association For Cancer Research 34: 491 3/93
	Helling, F., et al. "Construction of immunogenic GD-3 conjugate vaccines." August 1993 Annals Of The New York Academy Of Sciences

EXAMINER	DATE CONSIDERED
A. Gupta	3/28/97

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.

Philip O. Livingston and Friedhelm Helling
U.S. Serial No. 08/477,147
Filed: June 7, 1995
Exhibit 1

Form PTO-1449

INFORMATION DISCLOSURE CITATION IN AN APPLICATION

(Use several sheets if necessary)

Docket Number (Optional)	Application Number
43016-D/JPW/AKC/JKM	08/477,147
Applicant: Philip O. Livingston and Friedhelm Helling	
Filing Date: June 7, 1995	Group Art Unit: 1806

U. S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	690: 396-397
	Helling, F., et al. "GD-3 vaccines for melanoma superior immunogenicity
	of key hole limpet hemocyanin conjugate." 1994 Cancer Research 54:
	197-203

EXAMINER	DATE CONSIDERED
A. Caputa	3/29/97

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.

Form PTO-1449

INFORMATION DISCLOSURE CITATION IN AN APPLICATION

(Use several sheets if necessary)

Docket Number (Optional)	Application Number
43016-D/JPW/AKC/JKM	08/477,147
Applicant: Philip O. Livingston and Friedhelm Helling	
Filing Date: June 7, 1995	Group Art Unit: 1806

U. S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

✓	Bystryn, J-C, "Tumor vaccines." 1990, Cancer And Metastasis Reviews, 9: 81-91
✓	Ritter, G., et al. "Antibody response to immunization with ganglioside GD3 and GD3 congeners (lactones, amide and gangliosidol) in patients with malignant melanoma." 1991, Int. J. Cancer 48: 379-385

EXAMINER	DATE CONSIDERED
J. Epstein	3/28/97

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.

PROCEEDINGS

84th Annual Meeting of the American Association for Cancer Research

May 19-22, 1993 • Orlando, FL

CONTENTS

VOLUME 34 • MARCH 1993

amination. GD3 was conjugated to meningoccocal outer membrane proteins, cationized bovine serum albumin, multiple antigenic peptides, polylysine, and keyhole limpet hemocyanin. These proteins are known to be potent carriers for peptide or carbohydrate antigens. The GD3-protein conjugates were administered to mice with the adjuvant QS21, and antibody titers were determined by ELISA. Specificity of the immune response was further analyzed by dot blot immune stains on purified gangliosides and by immune thin layer chromatography using tumor tissue extracts. Of the GD3 conjugates tested, GD3 conjugated to keyhole limpet hemocyanin induced the strongest immune response. By contrast to the results of our previous trials with vaccines containing gangliosides plus adjuvants without conjugation, we are now able to induce more consistent, longer-lasting, higher titer IgM antibody response; in addition, we are able for the first time to induce high titer IgG responses. These results provide the basis for testing the immunogenicity of ganglioside-keyhole limpet hemocyanin conjugates plus QS21 in patients with melanoma.

[CANCER RESEARCH 54, 197-203, January 1,

G_{D3} Vaccines for Melanoma: Superior Immunogenicity of Keyhole Limpet Hemocyanin Conjugate Vaccines[1]

Friedhelm Helling,[2] Ann Shang, Michele Calves, Shengle Zhang, Shunlin Ren, Robert K. Yu, Herbert F. Oettgen, and Philip O. Livingston

Immunology Program and Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [F. H., A. S., M. C., S. Z., H. F. O., P. O. L.], and Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0614 [S. R., R. K. Y.]

ABSTRACT

Cell surface gangliosides show altered patterns of expression as a consequence of malignant transformation and have therefore been of interest as potential targets for immunotherapy, including vaccine construction. One obstacle has been that some of the gangliosides that are overexpressed in human cancers are poorly immunogenic in humans. A case in point is G_{D3}, a prominent ganglioside of human malignant melanoma. Using an approach that has been effective in the construction of bacterial carbohydrate vaccines, we have succeeded in increasing the immunogenicity of G_{D3} in the mouse by conjugating the ganglioside with immunogenic carriers. Several conjugation methods were used. The optimal procedure involved ozone cleavage of the double bond of G_{D3} in the ceramide backbone, introducing an aldehyde group, and coupling to aminolysyl groups of proteins by reductive amination. Conjugates were constructed with a synthetic multiple antigenic peptide expressing repeats of a malarial T-cell epitope, outer membrane proteins of *Neisseria meningitidis*, cationized bovine serum albumin, keyhole limpet hemocyanin, and polylysine. Mice immunized with these conjugates showed a stronger antibody response to G_{D3} than mice immunized with unconjugated G_{D3}. The strongest response was observed in mice immunized with the keyhole limpet hemocyanin conjugate of the G_{D3} aldehyde derivative and the adjuvant QS-21. These mice showed not only a long-lasting high-titer IgM response but also a consistent high-titer IgG response (predominantly IgG1), indicating recruitment of T-cell help, although the titers of IgM and IgG antibodies following booster immunizations were not as high as they are in the response to classical T-cell-dependent antigens. This method is applicable to other gangliosides, and it may be useful in the construction of immunogenic ganglioside vaccines for the immunotherapy of human cancers expressing gangliosides on their cell surface.

INTRODUCTION

Gangliosides are glycolipid constituents of the cell membrane. The term was coined in 1942 to refer to lipids of the central nervous system that contained sialic acid, to signify their prime location in ganglion cells and their glycosidic nature (1). Their lipophilic component, the ceramide (an amide-linked long-chain sphingoid base and a fatty acid), is thought to be embedded in the outer membrane of the cell membrane lipid bilayer. The carbohydrate portion of the molecule is oriented toward the outside of the cell. Malignant transformation appears to activate enzymes involved in ganglioside glycosylation, resulting in altered patterns of ganglioside expression in tumors such as astrocytoma, neuroblastoma, and malignant melanoma (2). In normal melanocytes, for example, the predominant ganglioside is G_{M3}.[3] Other gangliosides including G_{D3}, G_{M2}, G_{D1a}, and G_{T1b} constitute less than 10% of the total (3). In malignant melanoma, increased expression of G_{D3}, G_{D2}, and G_{M2} has been observed (4, 5), and these gangliosides have therefore been considered potential targets for immunotherapy.

One approach to ganglioside-targeted immunotherapy has been the use of mAbs.[4] Treatment of patients with melanoma or neuroblastoma with mAb recognizing G_{D3}, G_{D2}, or G_{M2} has resulted in tumor regression in some cases (6–9). The other approach has been to immunize patients with ganglioside vaccines in attempts to induce production of ganglioside antibodies by the patients themselves. These attempts have been successful so far only with G_{M2} vaccines. Patients with American Joint Committee on Cancer Stage III malignant melanoma, after complete resection of all tumor, have been shown to produce anti-G_{M2} antibodies in response to vaccination with G_{M2} and *Bacillus Calmette-Guérin* (after pretreatment with low-dose cyclophosphamide to reduce suppressor activity), and the disease-free interval and overall survival were longer in patients producing G_{M2} antibodies (10). G_{D3} and G_{D2}, on the other hand, were found to be only rarely immunogenic when administered in the same way to patients with melanoma (11). Even with the G_{M2} vaccines, the antibody response showed the characteristics of a T-cell-independent response, that is to say, IgM production of short duration, rare conversion to IgG production, and lack of a booster effect (12, 13).

Similar difficulties have been encountered in the development of effective vaccines against bacterial carbohydrate antigens. One approach that has been successful in overcoming these problems is conjugation of the antigen with immunogenic protein carriers. For example, a conjugate vaccine that links the *Haemophilus influenzae* type b capsular polysaccharide to the outer-membrane protein complex of *Neisseria meningitidis* serogroup B was recently shown to induce the production of antibodies and a high rate of protection against invasive disease caused by *Haemophilus influenzae* type b in infants (14), and similar results were reported for a conjugate vaccine using a nontoxic mutant diphtheria toxin as carrier (15).

We have explored this approach in attempts to increase the immunogenicity of melanoma gangliosides. We report here the effects of conjugating G_{D3} with several protein carriers on its immunogenicity in the mouse.

MATERIALS AND METHODS

Gangliosides. G_{M3}, G_{M2} and G_{D1b}, extracted from bovine brain, were provided by Fidia Research Laboratory (Abano Terme, Italy). G_{D2} was made from G_{D1b} by enzymatic cleavage with β-galactosidase from bovine testes (16). G_{D3} (mel) was isolated from human melanoma tissue (17), G_{D3} (bbm) and GT3 were isolated from bovine buttermilk (18), and disialyllactose (G_{D3} oligosaccharide) was isolated from bovine colostrum as previously described (19).

Reagents. HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol, *p*-nitrophenyl phosphate disodium, and sodium cyanoborohydride were from Sigma Chemical Co. (St. Louis, MO);

Received 7/30/93; accepted 11/1/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grants CA33049 and CA 08478, USPHS Grant NS-11853-19, and the Perkin Foundation.

[2] To whom requests for reprints should be addressed, at Immunology Program, Memorial Sloan-Kettering Cancer Center, 425 East 68th Street, Room 820 K, New York, NY 10021.

[3] The designations G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D1a} and G_{D1b} are used in accordance with the abreviated ganglioside nomenclature proposed by Svennerholm (40).

[4] The abbreviations used are: mAb, monoclonal antibody; MAP, multiple antigenic peptide; OMP, outer membrane protein; cBSA, cationized bovine serum albumin; ITLC, immune thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; ELISA, enzyme-linked immunosorbent assays; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; bbm, bovine buttermilk.

methylsulfide was from Aldrich (Milwaukee, WI); cyclophosphamide (Cytoxan) was from Mead Johnson (Syracuse, NY); and QS-21 adjuvant, a homogeneous saponin component purified from *Quillaja saponaria* Molina tree (20), was kindly donated by Cambridge Biotech Corp (Worcester, MA). It is an amphipathic molecule and was provided as a white powder, forming a clear colorless solution when dissolved in PBS.

Proteins. Poly-L-lysine hydrobromide [MW(vis)3800] was purchased from Sigma, keyhole limpet hemocyanin (KLH) was from Calbiochem (La Jolla, CA), the cBSA-Imject Supercarrier immune modulator was from Pierce (Rockfort, IL), and *Neisseria meningitidis* OMPs were kindly provided by Dr. M. S. Blake (Rockefeller University, New York). MAP YAL-IV 294-I containing four repeats of a malarial T-cell epitope was a gift from Dr. J. P. Tam (Rockefeller University).

Monoclonal Antibodies. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for ITLC, and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISAs, were obtained from Zymed (San Francisco, CA); anti-G_{D3} mAb R24 was generated in our laboratory (21).

Serological Assays. ELISA were performed as previously described (13). To control for nonspecific "stickiness," immune sera were also tested on plates to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAb or mouse sera was performed after separation on HPTLC silica gel glass plates as previously described (4). Plates were developed in solvent 1 [chloroform:methanol:water (0.25% $CaCl_2$), 50:40:10 (v/v)] or solvent 2 [ethanol:*n*-butylalcohol:pyridine:water:acetic acid, 100:10:10:30:3 (v/v)], and gangliosides were visualized with resorcinol-HCl reagent. Dot-blot immune stains were performed on nitrocellulose strips utilizing purified gangliosides spotted in equal amounts and developed as described before (13).

Immunization. Six-week-old female BALB/c × C57BL/6 F_1 mice (The Jackson Laboratory, Bar Harbor, ME) were given an i.p. injection of cyclophosphamide (15 mg/kg) 3 days before the first immunization and were then assigned to treatment groups. Groups of 4 or 5 mice were given three s.c. injections of a vaccine 2 weeks apart if not otherwise indicated. Each vaccine contained 20 μg G_{D3} or 15 μg disialyllactose and 10 μg QS-21 in a total volume of 0.1 ml PBS. Mice were bled from the retroorbital sinus before vaccination and 2 weeks after the last vaccine injection unless indicated otherwise.

G_{D3} Conjugate Preparation. G_{D3} (2 mg) was dissolved in 2 ml methanol by sonication and cooled to −78°C in an ethanol/dry ice bath. Ozone was generated in an ozone generator (Del Industries, San Luis Obispo, CA) and was passed through the sample for 30 min under vigorous stirring (22, 23). The excess of ozone was then displaced with nitrogen over a period of 10 min. Methylsulfide (100 μl) was added (24), and the sample was kept at −78°C for 30 min and then at room temperature for 90 min under vigorous stirring. The sample was dried under a stream of nitrogen and monitored by HPTLC. The long-chain aldehyde was separated by adding *n*-hexane (2 ml) to the dry sample, followed by sonication for 5 min and centrifugation at 2000 × *g* for 15 min. The *n*-hexane was carefully drawn off and discarded, and the sample was dried under a stream of nitrogen. Cleaved G_{D3} and native G_{D3} were separated by HPLC (Waters, System 501, Milford, MA) utilizing a C_{18} reversed-phase column (10 x 250 mm; Rainin Instruments, Ridgefield, NJ). Gangliosides were eluted with a linear water-acetonitrile gradient and monitored at 214 nm, and the fractions were analyzed by HPTLC. Fractions that contained cleaved G_{D3} were combined and evaporated at 37°C with a Rotavapor (Büchi, Flawils, Switzerland). Cleaved G_{D3} (1.5 mg), 1.5 mg protein carrier in PBS, and 2 mg sodium cyanoborohydride were incubated under gentle agitation at 37°C for 48 h. After 16 h 1 mg sodium cyanoborohydride was added. The progress of coupling was monitored by HPTLC. G_{D3}-protein conjugates did not migrate in solvent 1 and solvent 2 but remained at the origin as a resorcinol-positive band. The mixture was dialyzed across 5000 molecular weight cutoff dialysis tubing with three changes of PBS (4 liters each), at 4°C for 48 h, and passed through an Extractigel detergent-removing gel (Pierce, Rockfort, IL) for final purification of unconjugated G_{D3}. The samples were lyophilized, and their protein and ganglioside content was determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).

Disialyllactose Conjugate Preparation. Disialyllactose was isolated from bovine colostrum as described previously (19). The carbohydrate was attached to protein by reductive amination (26). Disialyllactose (10 mg) was incubated with 2 mg proteins in 2 ml PBS for 14 days at 37°C after sterile filtration. Sodium cyanoborohydride (2 mg) was added at the beginning, and 1 mg was added every 3 days. The coupling was monitored by HPTLC in solvent 2. The disialyllactose conjugates were purified by dialysis across 5000 molecular weight cutoff dialysis membrane with three changes of PBS (4 liters each) at 4°C for 48 h, followed by lyophilization. The protein and neuraminic acid content was determined as described above. Disialyllactose was also conjugated to proteins according to the method described by Roy and Laferrière (27). During this procedure *N*-acroloylated glycopyranosylamine derivatives of the oligosaccharide were formed first, followed by conjugation via Michael addition to amino groups of the protein. Purification and determination of protein and neuraminic acid content were performed as described above.

Determination of Antibody Subclasses. Determination of antibody subclasses was performed by ELISA using subclass-specific rabbit anti-mouse immunoglobulins IgG1, IgG2a, IgG2b, IgG3, and IgA (Zymed, San Francisco, CA). Alkaline phosphatase-labeled goat anti-rabbit IgG served as the signal-generating reagent.

FACS Analysis of Mouse Antisera. A single cell suspension of the melanoma cell line SK-MEL-28 was obtained after treatment with 0.1% EDTA in PBS followed by passage through a 26½-gauge needle. Cells (3×10^5) were incubated with 40 μl of 1:20 diluted post- or preimmunization serum for 30 min on ice. The cells were washed three times with 3% fetal calf serum in PBS. Thirty μl of diluted (1:50) fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) were added as secondary antibody, followed by incubation on ice for 30 min. Cells were washed three times as above and resuspended in 500 μl 3% fetal calf serum in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

RESULTS

Preparation and Characterization of G_{D3}-Protein Conjugates. G_{D3} (bbm) in methanol was selectively cleaved with ozone at the C4-C5 double bond in the ceramide portion. It is assumed that methoxyperoxides are formed as intermediate products (24), and therefore methylsulfide was added as a reducing agent. The result of the cleavage was a G_{D3} derivative with an aldehyde functional group in the position of the former double bond in the ceramide portion (Fig. 1). Cleaved G_{D3} migrated slower than native G_{D3}, and formed double bands because the ceramide contained unsaturated fatty acids that were cleaved simultaneously (see Fig. 1, *inset*). Densitometric analysis of HPTLC plates showed that more than 70% of G_{D3} (bbm) was cleaved by this procedure. Preliminary experiments involving longer ozone treatment had similar results, indicating that 30% of G_{D3} from this source consists of sphinganine or phytosphingosine analogues that contain no ozone-cleavable ceramide double bond. Cleavage at −78°C with ozone treatment up to 1 h (depending on the amount of G_{D3} used) was found to be optimal. Cleaved G_{D3} persisted only in acidic and neutral phosphate buffers for up to 72 h, but with the formation of increasing amounts of oligosaccharide due to β-elimination reactions [which have been shown to occur much faster at alkaline pH (23)]. The decreased hydrophobicity of cleaved G_{D3} compared to native G_{D3} allowed its separation by HPLC on C_{18} reversed-phase columns. Utilizing isocratic elution with a linear water-acetonitrile gradient, cleaved G_{D3} was recovered first, and uncleaved G_{D3} was eluted in later fractions. The incubation of cleaved G_{D3} with proteins resulted in the formation of Schiff bases between the cleaved ganglioside and ε-aminolysyl groups. They were reduced with sodium cyanoborohydride to form stable secondary amine bonds (28). The reaction was monitored by HPTLC, which showed a decreasing ratio of the cleaved G_{D3} to a resorcinol positive band at the origin, indicating the formation of neoglycoconjugates. The reaction was generally completed after incubation for 48 h at 37°C. Disialyllactose was readily remov-

Construction of Immunogenic GD3-Conjugate Vaccines

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Gangliosides are sialic acid-containing glycosphingolipids composed of a carbohydrate moiety linked to a hydrophobic ceramide portion. Whereas ceramide is embedded within the outer leaflet of the cell membrane, the carbohydrate chain is exposed to the extracellular matrix. Qualitative and quantitative changes in ganglioside composition during cell differentiation and proliferation have been observed and seem to reflect the state of malignant transformation of cancers of neuroectodermal origin. Malignant melanoma cells express a variety of gangliosides in addition to GM3, the major ganglioside in normal melanocytes. Altered ganglioside metabolism of transformed cells causes additional expression of GD3, the major ganglioside in melanoma, and GM2, GD2, 9-O-acetyl-GD3, and GT3.

We vaccinated patients with a series of whole cell melanoma vaccines selected for expression of a variety of glycoprotein and ganglioside antigens and found that the only antigens recognized by more than one patient were the gangliosides GM2 and GD2. When vaccines containing purified gangliosides are used in melanoma patients, GM2/BCG vaccines seemed to be most effective. In a randomized study of 122 melanoma patients who were disease-free after surgery, we showed that most patients (86%) receiving the GM2 vaccine produced antibodies. Patients who produced anti-GM2 antibodies had a significantly longer disease-free and overall survival than did antibody-negative patients. In comparing the two arms of the trial, we found that patients receiving the GM2/BCG vaccine had a 17% improvement in disease-free interval and a 9% improvement in survival compared with the bacillus Calmette-Guérin control group, although neither result was statistically significant. Unfortunately, the immune response was only of short duration, mostly IgM, and of moderate titer. This suggested that GM2 was recognized as a T-cell–independent antigen because gangliosides are carbohydrate antigens and also because gangliosides are autoantigens expressed on some normal tissue. Similar approaches with GD2 and 9-O-acetyl-GD3 vaccines in patients resulted in occasional low titers, and no antibody response against GD3 could be detected.

A different approach had to be utilized; therefore, we explored whether covalent attachment to immunogenic carriers, an approach effective with bacterial carbohydrate antigens, would increase the immunogenicity of GD3. Conjugates containing solely the oligosaccharide part of GD3 were not reactive with mAb R24 and were unable to induce a significant immune response against GD3 ganglioside. Modification of glucose at the reducing end of the oligosaccharide chain during conjugation or the missing part of the ceramide may influence proper epitope presentation and detection by the immune system. Our second approach was aimed at coupling GD3 via its ceramide portion without altering the carbohydrate part. GD3 was subjected to ozone cleavage of the ceramide double bond and the resulting aldehyde group was coupled to amino groups of proteins by reductive

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Increased immunogenicity of GM2 conjugated with KLH and used with adjuvants in patients with melanoma. Helling F., Adluri S., Calves M., Koganty R.R., Oettgen H.F., Livingston P.O., Memorial Sloan-Kettering Cancer Center, New York, NY 10021 and Biomira Inc., Edmonton, Alberta, Canada.

In a series of studies aimed at inducing an antibody response in melanoma patients to gangliosides expressed by melanoma we have shown that immunization with GM2 and BCG elicits a brief IgM response in most patients, but only rarely IgG. One of the approaches we have taken to increase ganglioside immunogenicity is conjugation with immunogenic protein carriers. We report that conjugation of GM2 with keyhole limpet hemocyanin (KLH) results in increased immunogenicity in Stage III melanoma patients, and that use of the saponin-type adjuvant QS21 (Cambridge Biotech, Worcester, MA) further enhances this effect. Median IgM titers by ELISA after vaccination were: GM2 0, GM2-KLH 1/80, GM2-KLH/BCG 1/160, GM2-KLH/DETOX 1/160 and GM2-KLH/QS21 1/1280. The antibody response to GM2-KLH/QS21 lasted longer, and IgG antibodies were consistently induced (median titer 1/160). This approach can now be extended to GD2 and GD3, major melanoma gangliosides of poor immunogenicity. Supported by NIH grant CA 40532.

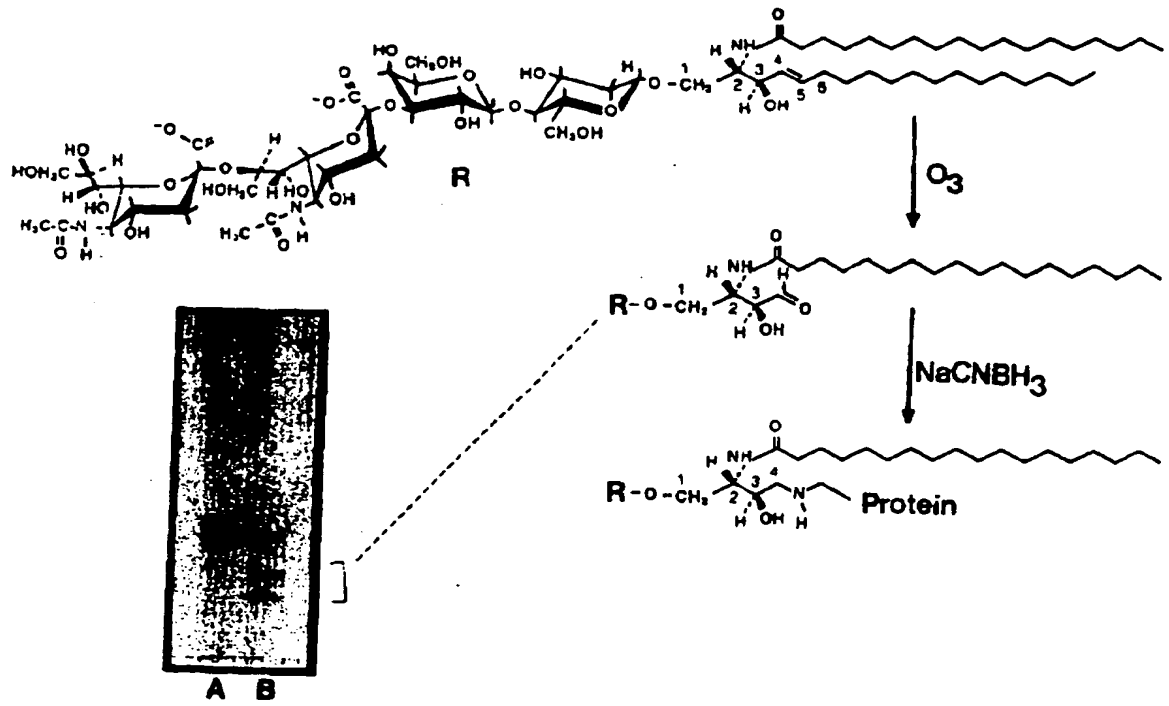

Fig. 1. Synthesis of G_{D3} protein conjugates after ozone cleavage and reductive amination. *Inset*, HPTLC of G_{D3} before (*A*) and after (*B*) ozone cleavage.

able by dialysis, and the excess of cleaved G_{D3} was removed by passage through a detergent-removing column. The degree of coupling was determined by sialic acid and protein determinations. The weight ratio of G_{D3} to proteins in the different conjugates, shown in Table 1, depended on the accessibility of lysine groups in the proteins. The average yield of G_{D3} coupled to proteins was 30%. G_{D3} conjugates prepared in this way were reactive with anti-G_{D3} mAb R24 by Western blot analysis, although the G_{D3}-aldehyde derivative itself was not reactive by ITLC (data not shown).

Oligosaccharide Conjugation. The carbohydrate part of G_{D3}, disialyllactose, was coupled to proteins utilizing two methods. The first method, reductive amination, resulted in conjugation of the open ring form of the glucose to proteins (26). The method required a long incubation of the oligosaccharide with proteins, and the yield was less than 20%. In the second method (27), involving *N*-acroloylation of the terminal glucose, the oligosaccharide was coupled to proteins with a closed ring formation. None of these oligosaccharide conjugates showed reactivity with mAb R24 by Western blot analysis (data not shown).

Induction of a Serological Response against G_{D3} by Immunization with G_{D3}-Protein Conjugates. All vaccines were well tolerated. Mice were observed for at least 6 months, and neither acute nor systemic toxicity was detected. The serological response to immunization with G_{D3} or G_{D3}-protein conjugates, using QS-21 as adjuvant, is shown in Table 1. QS-21 was used because we had previously demonstrated its superiority over other adjuvants with another carbohydrate antigen-KLH conjugate vaccine (29). In ELISA, preimmunization sera showed no IgM or IgG antibodies reactive with G_{D3}. Immunization with unconjugated G_{D3} did not induce the production of G_{D3} antibodies. Immunization with G_{D3} conjugates, on the other hand, was effective in inducing antibody production. Of the five proteins used in the preparation of the conjugates, KLH showed the

Table 1 *Antibody response to immunization with different vaccines containing G_{D3} or disialyllactose conjugated to carrier proteins*

Vaccine + QS-21	No. of mice	G_{D3}:protein weight ratio[a]	Reciprocal ELISA peak titer against G_{D3}	
			IgG	IgM
G_{D3}	5		0 (5)	20 (3), 0 (2)
G_{D3}/KLH[b]	5	0.33	0 (5)	160, 40, 20 (3)
G_{D3}-KLH[c]	14	0.69	10,240 (2), 5,120 (2), 2,560 (3), 1,280 (2), 80 (2), 40 (2), 0	2,560, 1,280 (2), 640, 320 (3), 160 (2), 80 (3), 20, 0
G_{D3}-cBSA[c]	15	0.77	2,560 (2), 320 (2), 160, 80 (2), 40 (4), 20 (2), 0 (2)	80 (2), 40 (2), 20 (7), 0 (4)
G_{D3}-OMP[c]	15	0.93	2,560, 80 (4), 20 (3), 0 (7)	1,280, 320 (2), 160 (7), 80 (4), 40
G_{D3}-MAP[c]	10	1.0	40, 0 (C)	160 (2), 40 (4), 20 (3), 0
G_{D3}-Polylysine	10	ND	0 (10)	320, 160 (4), 80, 40, 20 (2), 0
Disialyllactose-KLH[d]	4	0.055	0 (4)	160 (3), 80
Disialyllactose-cBSA[d]	4	0.16	20, 0 (3)	40, 20 (3)
Disialyllactose-KLH[e]	4	0.25	20, 0 (3)	40 (2), 0 (2)
Disialyllactose-cBSA[e]	4	0.34	0 (4)	0 (4)
Disialyllactose-Polylysine	5	ND	0 (5)	80 (3), 40 (2)

[a] Protein and ganglioside content were determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).
[b] G_{D3} and KLH were mixed prior to immunization.
[c] G_{D3} was covalently attached to proteins prior to immunization after ozonolysis as described in "Materials and Methods."
[d] Disialyllactose was conjugated to KLH and cBSA by reductive amination according to the method of Gray (26).
[e] Disialyllactose was conjugated to KLH, cBSA, and poly-L-lysine after *N*-acroloylation and Michael addition according to the method of Roy and Lafferière (27).

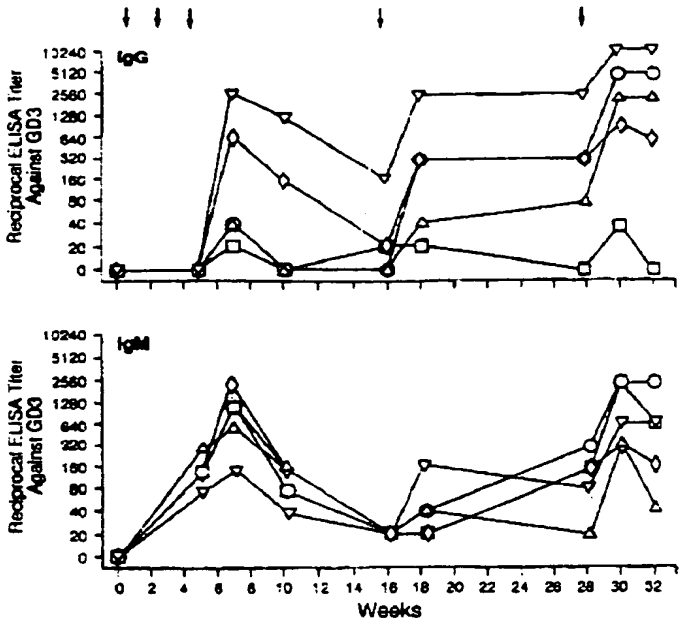

Fig. 2. Time course of G_{D3} antibodies induced in representative mice immunized with G_{D3}-KLH and QS-21 vaccine. Each symbol represents an individual mouse. Arrows, time of vaccination.

strongest immunogenicity, resulting in a median titer of 1:320 for IgM and 1:2560 for IgG antibodies. The specific isotype profile was determined with subclass-specific secondary rabbit anti-mouse antibodies. Antigen-specific antibodies were found to be predominantly of the IgG1 subclass. Antigen-specific IgG2a and IgG2b antibodies were found only in traces, and no IgG3 or IgA antibodies were detected.

In contrast to immunization with G_{D3} conjugates, immunization with G_{D3}-oligosaccharide conjugates induced only a weak IgM response to G_{D3} and no IgG response.

Sequential IgM and IgG antibody titers against G_{D3} for five mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 2. IgM titers peaked 2 weeks after the third vaccination and declined by the time of the first booster immunization at week 16. The first booster immunization had no significant impact on IgM titers, but the second booster immunization at week 28 increased IgM titers to the peak level seen after the third vaccination of the initial series. IgG titers also rose up to 2 weeks after the third vaccination and decreased by the time of the first booster vaccination but rapidly increased after the booster to previous peak titers. IgG titers remained at this level for 10 weeks, with a further increase after the second booster in most mice. The evidence for a secondary immune response after the booster immunization was therefore equivocal. The response was clearly more rapid than after the initial immunization and lasted longer, but the increase in titer was not comparable to booster responses seen with classical T-cell-dependent antigens.

Specificity of the Serological Response to Immunization with G_{D3}-Protein Conjugates. The specificity of the serological response to immunization with G_{D3}-protein conjugates and QS-21 was analyzed by dot-blot immune staining and ITLC. An example of dot-blot immune stain analysis is shown in Fig. 3. Preimmune sera and immune sera showing high G_{D3}-antibody titers in ELISA were tested on nitrocellulose strips that had been spotted with G_{D3} (bbm) or G_{D3} (mel) and purified structurally related gangliosides: G_{M3}, G_{D2}, G_{D1b}, and G_{T3}. As expected on the basis of the ELISA results, preimmune sera showed no reactivity. In contrast, sera obtained after immunization with KLH conjugates of G_{D3}-ganglioside reacted with G_{D3} (bbm) (the immunogen) or G_{D3} (mel), but not with the other gangliosides except G_{T3} in some cases, a pattern also seen in tests of the mouse monoclonal IgG3 antibody R24, the reagent by which high cell surface expression of G_{D3} on human melanoma cells was first defined (20). The same specificity pattern was seen in dot-blot immune stain tests of sera from mice immunized with other G_{D3}-protein conjugates, the only exception being high-titer sera (by ELISA) from mice immunized with G_{D3}-cBSA, which showed no reactivity with G_{D3} or the other gangliosides.

ITLC permits specificity analysis of ganglioside antibodies in tests on tissue extracts. Examples of tests with high-titer sera from mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 4. The sera were tested at a dilution of 1:150 on ganglioside extracts of human brain, neuroblastoma, and melanoma, as well as G_{D3} (bbm) that had been used for immunization. The figure shows HPTLC ganglioside patterns of these reagents after staining with resorcinol, as compared with the patterns of reactivity exhibited after exposure to sera from immunized mice or mAb R24. As can be seen in the resorcinol-stained panel, the predominant gangliosides in the brain tissue extract are G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}, whereas the neuroblastoma extract shows G_{D2} and G_{M2} in addition, and the melanoma extract contains mainly

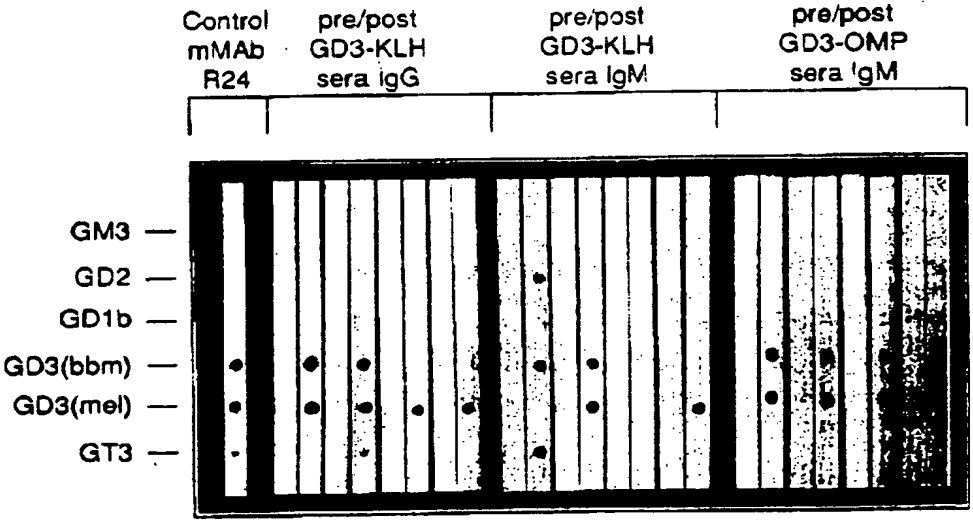

Fig. 3. Dot-blot immune stain assay for IgM and IgG antibodies in sera of mice immunized with G_{D3}-KLH and G_{D3}-OMP conjugates and QS-21. Antigen standards were applied to nitrocellulose strips in equal amounts (0.5 μg) and were allowed to react with pre/postimmunization serum from individual mice.

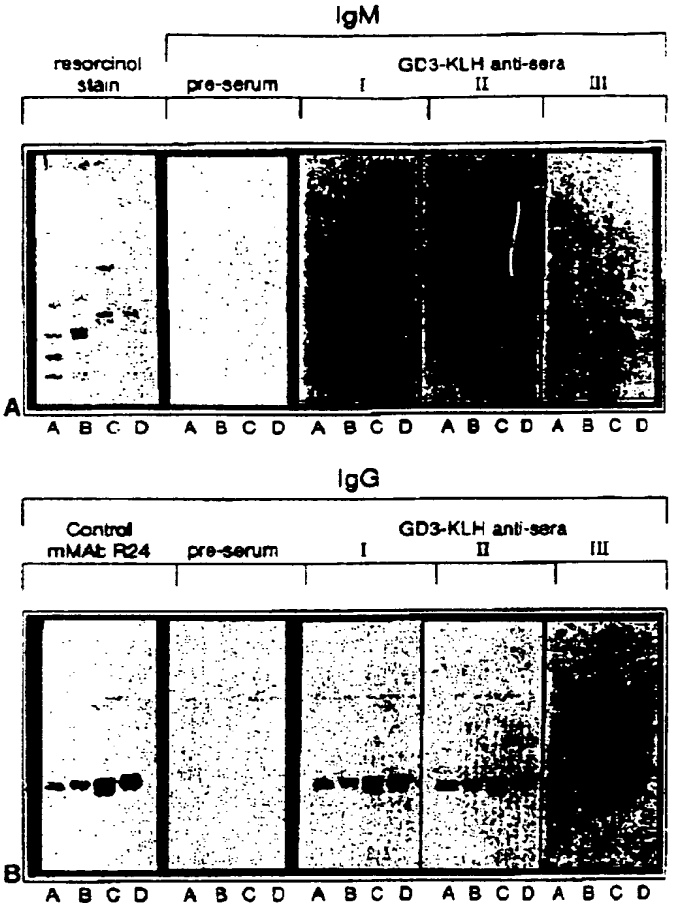

Fig. 4. Immune thin-layer chromatograms of three representative mouse sera after vaccination with G_{D3}-KLH conjugate and QS-21. IgG and IgM antibodies in pre- and postvaccination sera and anti-G_{D3} mMab R24 were tested on human brain gangliosides (*A*), neuroblastoma gangliosides (*B*), melanoma gangliosides (*C*), and G_{D3} (*D*) (bbm). Gangliosides were chemically stained with resorcinol-HCl reagent to demonstrate the ganglioside composition of each sample.

G_{D3} and G_{M3}. Reactivity of IgG antibodies in postimmunization sera, as well the reactivity of IgG3 mouse monoclonal antibody R24, was restricted to G_{D3} (Fig. 4*b*). The high-titer IgM antibodies, on the other hand, showed weak cross-reactivity with other gangliosides and sulfatide in the brain extract (Fig. 4*a*).

Sera from mice immunized with other G_{D3} conjugates were tested in the same way (at lower dilution) and showed the same specificity with the exception, again, of high-titer sera from mice immunized with G_{D3}-cBSA, which showed no ganglioside reactivity (data not shown).

Cell Surface Reactivity of Immune Sera Determined by FACS Analysis. Sera from mice were tested for binding to cells of the melanoma cell line SK-MEL-28, a cell line known to express cell surface G_{D3}. A representative example of a FACS analysis utilizing a fluorescein isothiocyanate-labeled secondary goat anti-mouse antibody is shown in Fig. 5. Sera before and after immunization with G_{D3}-KLH and QS-21 were tested. Preimmunization serum stained 8% of the target cells, postimmunization serum 92%.

DISCUSSION

Conjugation of poorly immunogenic antigens to highly immunogenic carrier molecules is a well-known approach to augmenting immunogenicity. Ganglioside molecules are so small, however, that linkage to carrier molecules without affecting the relevant antigenic epitopes is difficult. We have shown previously that modifications of G_{D3} in its carbohydrate portion (*i.e.*, conversion of sialic acid carboxyl groups to amides or gangliosidols or lactones) results in markedly increased immunogenicity. However, antibodies produced in response to these G_{D3} derivatives show no cross-reactivity with native G_{D3} (11, 30). Covalent attachment of proteins to the sialic acid molecules of G_{D3} was therefore not attempted in the present study. Our initial approach involved conjugation of G_{D3} oligosaccharide (disialyllactose) via the terminal glucose in open- or closed-ring configuration to KLH or polylysine, but these conjugates were not recognized by the anti-G_{D3} mAb R24 or by mouse antisera to G_{D3}, and mice immunized with the conjugates did not produce G_{D3} antibodies. Subsequently, we coupled G_{D3} to proteins via its ceramide portion without alteration of the carbohydrate moiety. The ceramide was cleaved with ozone at the double bond of the sphingosin base, and coupling to proteins was accomplished by reductive amination. Cleavage of gangliosides by ozonolysis and subsequent conjugation with proteins by this method has not been described, and it has been generally assumed that the aldehyde intermediates of gangliosides would be unstable. Fragmentation, initiated by hydroxy ions under alkaline conditions, has been reported. Migration of the double bond would result in β-elimination, causing release of the oligosaccharide moiety (22, 31). We found, however, that the aldehyde was sufficiently stable at neutral pH to permit Schiff base formation with amino groups of proteins, so that β-elimination was not a major problem. The overall yield was 30%. These G_{D3} aldehyde-protein conjugates showed reactivity with G_{D3} antibodies by Western blot analysis, indicating that the immunodominant epitopes were intact in these G_{D3} conjugates. However, reactivity of the G_{D3}-aldehyde derivative with mAb R24 by ITLC could not be shown. This may be due to its relatively unstable nature, resulting in β-elimination and release of oligosaccharide during the immune stain incubation period, or simply to the fact that the G_{D3}-aldehyde derivative may not adhere to the thin-layer plate sufficiently for serological detection.

Earlier studies describe oxidative ozonolysis of the glycosphingolipid olefinic bond, resulting in a carboxyl group that could be conjugated with carbodiimide to NH_2 groups of modified glass beads, agarose gel, or other macromolecules (32, 33). This method, however, is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacetylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Once the conjugation method was established, several protein carriers were considered, based on previous work by others. Lowell *et al.* (34) described an elegant system that resulted in high-titer antibody responses as a consequence of anchoring bacterial carbohydrate and peptide antigens via a synthetic, hydrophobic foot in OMPs of *Neisseria meningitidis* (35). This system was directly applicable to gangliosides because of their amphipathic nature. In previous studies, we adsorbed gangliosides onto OMP by hydrophobic interaction, and we were able to induce high-titer IgM responses (36). Covalent attachment was utilized in the current study, but G_{D3}-OMP conjugates induced only occasional IgG responses, and the IgM response was not increased. Conjugation with cationized BSA, which has been reported to be a potent carrier for protein antigens (37), resulted in high-titer IgG antibodies detected by ELISA, but immune stains indicated that the response was not G_{D3}-specific. Another appealing carrier is the MAP system described by J. P. Tam (38, 39). MAPs consist of four or eight dendritic peptide arms, containing B- and T-cell epitopes, attached to an oligomeric branched lysine core. The antibody response to peptides was dramatically increased when these constructs were

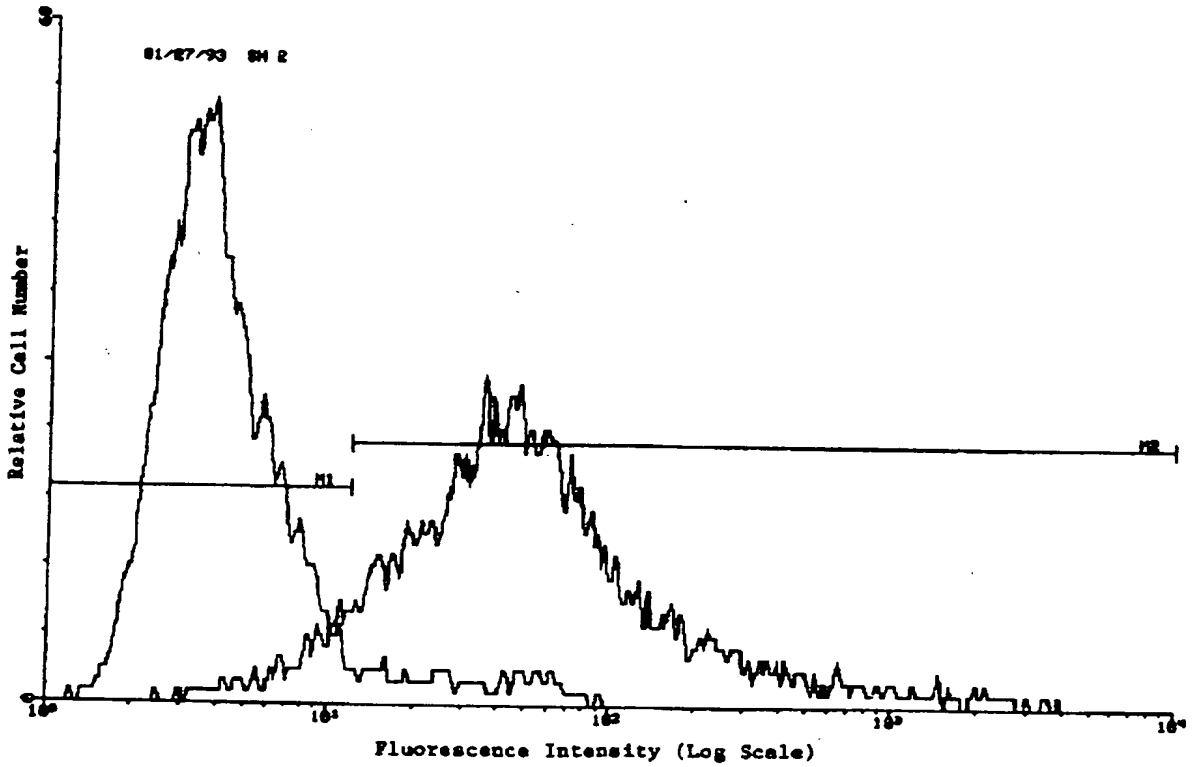

Fig. 5. Representative FACS analysis of mouse serum reactivity prior to (peak at 3) and after (peak at 50) immunization with G_{D3}-KLH and QS-21 tested on melanoma cell line SK-MEL-28.

used. When we attached G_{D3} to the amino terminal end of the MAP structure containing a malarial T-cell epitope, only a moderate IgM response against G_{D3} was detected, and there was no detectable IgG response. Conjugation of G_{D3} to polylysine resulted in a medium-titer IgM response and no IgG response, despite the high density of G_{D3} epitopes on these constructs.

The carrier that proved to be most effective in enhancing the antibody response to G_{D3} in this series was KLH. Immunization with G_{D3}-KLH consistently induced long-lasting production of IgM and IgG antibodies against G_{D3} at high titers. In comparing KLH with cBSA, OMP, MAP, and polylysine, it is difficult to know exactly why KLH is a superior carrier for G_{D3}. The sheer size and antigenic complexity of KLH stand out as a possible aid to antigen processing and recruitment of T-cell help across a broad range of T-cell specificities. The very qualities that make KLH cumbersome to work with are probably responsible for its unique effectiveness as a carrier in conjugate vaccines. KLH has not been widely used as a carrier for conjugate vaccines in humans because its size and heterogeneity make vaccine construction and standardization difficult.

Our hope was that conjugate vaccines would convert the T-cell-independent response against unconjugated G_{D3} seen in our previous studies t a T-cell-dependent response producing high-titer, long-lived, IgG antibodies. This expectation was fulfilled to some extent but not completely. The peak of the IgM response occurred after the third biweekly vaccination as in our previous studies with unconjugated G_{D3}, but the antibody titers were significantly higher. The response declined rapidly (as observed before), and additional vaccinations increased IgM titers to previous peak levels. The repeated increase in the titer of IgM antibodies to G_{D3} after booster immunizations differs from the expected response to T-cell-dependent antigens such as proteins, which generally induce little or no IgM response after booster immunizations. For the first time, however, we were able to induce a high-titer IgG response against G_{D3} ganglioside consistently. This response lasted significantly longer than the IgM response and was increased by additional vaccinations, although the response following booster vaccinations was not comparable to the exponential increase often seen with protein antigens. The fact that the G_{D3} antibodies were of the IgG1 subclass indicates that a T-cell-dependent pathway was activated by the G_{D3}-KLH conjugate vaccine. The lack of a classical booster effect, however, may reflect the carbohydrate nature of G_{D3} and its status as an auto-antigen. This suggests that T-cell recruitment by ganglioside conjugate vaccines is limited by the nature of the antigen itself. Nevertheless, the high-titer IgM response and long-lived IgG response to vaccination with G_{D3}-KLH and QS-21 seen in these experiments represents a striking improvement over the response to unconjugated ganglioside vaccines and can now form the basis for clinical trials of ganglioside-KLH conjugate vaccines in patients with cancers that show increased ganglioside expression.

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Tumor vaccines

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Abstract

Melanoma vaccines are an exciting and increasingly attractive immunotherapeutic approach for malignant melanoma. Vaccines can be used for patients with high risk primary melanoma and regional disease, stages in the progression of melanoma for which there is presently no treatment. They are unique in their potential to prevent cancer in high risk individuals.

Multiple approaches are being followed to develop effective vaccines. It is too early to judge whether any of them effectively slow the progression of melanoma. However, it is clear that vaccines are safe to use, and that they can stimulate immune responses to melanoma in some patients. The specificity of these responses needs to be clarified, and multiple challenges remain to be overcome before effective vaccines to melanoma become available. We must first identify the antigens on melanoma that stimulate immune responses, define the immune effector mechanisms that are stimulated by vaccine immunization and identify those responsible for increasing resistance to tumor growth, devise appropriate ways of constructing vaccines that will induce such responses, and find adjuvants and/or immunodulators that will potentiate desirable immune responses.

Introduction

Active specific immunotherapy with vaccines constructed of tumor antigens is a conceptually attractive approach to treat and possibly prevent cancer. The rationale is that such vaccines may be able to stimulate the immune system to react more vigorously, specificially to cancer cells and thus augment resistance to a patient's tumor. The most convincing evidence that this approach is valid is the fact that tumor vaccines prevent some cancers in animals [1–3]. Preliminary studies suggest that they may slow the progression of some cancers in humans [4–12].

Tumor vaccines have several potential advantages over other immunotherapies for cancer. They can selectively augment immunity to tumor, and thus should have a more potent effect than immunotherapeutic approaches that non-specifically augment immune reactivity, such as BCG, IL-2, and other immunomodulatory and biological response modifying agents. The immunity induced by vaccines has the potential to be long-lasting, as opposed to passive immunotherapy with monoclonal antibodies or adoptive therapy with immune cells, whose effect is transient. Vaccines can stimulate cellular responses, which are thought to be the principal effector mechanism in immune resistance to cancer. Vaccines are relatively non-toxic, so they can be used early in cancer progression, after the surgical removal of the primary tumor, at a stage when chemotherapy and other forms of immunotherapy are not used because of their toxicity. Finally, vaccines are unique in their potential for preventing cancer.

As a result, there is now an increasing interest in the use of vaccines to treat cancer. Much of it is focused on melanoma because it appears to be an

excellent model for the study of cancer vaccines [13]. This review analyzes the current status of cancer vaccines, using our efforts to develop a vaccine for human malignant melanoma as an example.

Rationale for cancer vaccines

It is important to realize that the progression of cancer depends not only on the instrinsic malignant potential of the tumor, but also on the patient's immune response to it. As a consequence, it may be possible to increase resistance to cancer by stimulating specific anti-tumor immunity with a vaccine.

In the case of malignant melanoma, the evidence that immune mechanisms may increase resistance to this cancer consists of the following observations:

1. The growth of melanoma in humans is influenced by host factors. The most dramatic demonstration of this is the spontaneous and complete regression of disseminated melanoma, a rare but well-documented phenomenon that occurs in up to 0.5% of patients [14]. Less dramatic but much more common is the partial regression of melanoma, an event which can be seen in up to 25% of primary melanomas [15]. There can be a prolonged latent period between the removal of a primary tumor and the appearance of metastatic lesions; during this time tumor cells are present in the body, but are prevented from growing by some mechanism [14]. Melanoma cells may circulate in the blood without metastatic spread, and conversely, metastatic disease may be present without a known primary [14], suggesting that the cancer cells, metastatic and primary respectively, have been destroyed. All of these events indicate that host factors can slow tumor growth and even cause regression. Unfortunately, these factors are not sufficiently potent to be able to prevent ultimate disease progression in most cases.

Two experiments of nature clearly demonstrate that the body has mechanisms available to achieve the goal of melanoma immunotherapy, i.e., the selective destruction of pigment cells. These are vitiligo and the 'halo' nevi phenomenon, conditions in which pigment cells are selectively destroyed without damage to nearby unrelated cells. The cause of pigment cell destruction in these conditions is unknown, but it is suspected that an anti-pigment cell immune response is the common denominator [16]. The presence of hypopigmentation favorably influences the prognosis of melanoma [17]; this suggests that the host mechanisms that selectively destroy normal pigment cells can also slow the progression of melanoma.

2. Tumors including melanoma cells can express antigens that differ qualitatively and/or quantitatively from those on normal adult tissues [18]. These antigens are sufficiently different from those on normal adult cells to be recognized as foreign and to trigger humoral and/or cellular immune responses in persons with cancer. At least in some cases, these responses have the capacity to destroy cancer cells *in vitro* [19].

3. Stimulation of anti-tumor immunity can increase resistance to tumor, while immunosuppression can enhance tumor growth. As indicated below, active immunization to melanoma vaccines can prevent melanoma in mice [1, 20]. Conversely, immunosuppression of animals by thymectomy or with anti-lymphocyte serum leads to more aggressive melanoma growth. The same is true in humans. The incidence of melanoma in immunosuppressed patients is four to eight times greater than one would expect [21]. These observations indicate that immune mechanisms have a real impact on tumor growth, rather than being interesting but clinically irrelevant epiphenomena, and that they can be manipulated to give clinically desirable results.

Vaccines prevent cancer in animals

The most convincing evidence that vaccines can increase resistance to cancer is that they prevent some tumors in animals [2, 3, 22, 23]. For example, we have shown that mice actively immunized to vaccines composed of whole B16 melanoma cells or to soluble, partially purified antigens extracted from the tumor will be protected against a challenge of syngeneic B16 melanoma cells that rapidly

kills all non-immunized mice [1]. This observation has been confirmed by others [20, 24]. The protective effect is specific [1], indicating that it is mediated by an immune mechanism.

Tumor protective immunity is associated with the development of specific antibodies and a cellular (delayed type hypersensitivity) immune response to B16 melanoma [1, 25]. The antibodies are directed to several surface antigens that are selectively expressed by B16 melanoma cells [25].

It is difficult to induce the rejection of already established tumors with vaccines [24]. However, this can be accomplished if tumor mass is reduced by surgery or chemotherapy, or if immunotherapy is initiated soon after tumor inoculation.

The implication of these findings is that tumor antigen vaccines may be able to increase resistance to cancer in humans, and that the approach will be most effective in patients with minimal disease. This, in turn, suggests that vaccines should be used early in the evolution of tumors or after tumor load has been reduced by surgery or chemotherapy. The clear ability of vaccines to prevent cancer in animals suggests that ultimately the most effective applications of vaccines may prove to be the prevention of this cancer in high-risk individuals.

Key issues in design of cancer vaccines

The ideal cancer vaccine should be safe, effective against a broad range of tumors of the same histological type, sufficiently potent to require only a few immunizations, simple to manufacture in a reproducible manner, and stable for a prolonged period of time. No such vaccine is available. Some of the key issues that must be considered in the construction of cancer vaccines are discussed below.

1. Selection of tumor antigen

In order to be effective, vaccines should be constructed from tumor antigens that are: a) able to induce clinically effective immune responses in humans; b) expressed on the tumor to be treated; and c) located at a site on the tumor where they can be seen by, and can interact with, immune effector mechanisms; i.e., on the external surface of tumor cells. Antigens that satisfy these requirements have not yet been identified.

2. Tumor antigen immunogenicity

The most basic requirement for a cancer vaccine is that it contains tumor antigens that can stimulate a strong and clinically effective antitumor immune response in humans. Little is known about the identity of such antigens. Most tumor antigens have been defined with monoclonal antibodies that are raised by immunizing animals with human tumor cells. These identify antigens that are recognized as foreign in animals, but they provide no information about the immunogenic potential of the antigen in humans. It is possible to identify tumor antigens that are immunogenic in humans by using human monoclonal antibodies, allogenic or autologous antisera, or human T cell clones. By applying these techniques, several antigens on malignant melanoma that are immunogenic in humans and cross reactive among melanomas have recently been identified. These include the gangliosides GD2, O-acetylated GD-3, and GM-2, a 250 kD glycoprotein, a urinary tumor associated antigen, and a 48 kD antigen. We have used immunoprecipitation SDS-PAGE analysis to compare the pattern of melanoma antigens defined by pre- and post-vaccine treatment sera in the same patients to directly identify antigens that can induce immune responses in humans. These have turned out to be cell surface antigens with molecular weights of 200+, 150, 110, 75, and 38 kDs [26]. The 200+ and 110 kD antigens are of particular interest for vaccine construction because they appear to be the most immunogenic, and seem to be selectively expressed on melanoma [27]. The functional effect of the immune responses induced by these antigens is still not known. Further application of these approaches will hopefully lead to the definition of panels of immunogenic tumor antigens that are broadly expressed on different melanomas and can be used to construct purified second generation vaccines.

3. Tumor antigen heterogeneity and modulation

For vaccine immunotherapy to be effective, the immune responses induced must be directed to antigens expressed by the tumor being treated. Unfortunately, the pattern of tumor antigens expressed by cancers of the same histological type in different individuals is variable. There is also variation in the pattern of tumor antigens expressed by different tumor nodules in the same individual, and by different tumor cells in the same nodule [28]. In addition, as a result of antigenic modulation, the profile of tumor antigens expressed by a tumor during its progression may be altered by the immune response of the host [29]. As a consequence, it is unlikely that vaccines prepared from a single tumor antigen will be effective against a broad range of tumors of the same histological type. For the same reason preparing autolegors vaccines from a patients own tumor cells does not ensure it will be effective against other tumor cells in the same patient. However, many tumor antigens are commonly expressed on many, although not all, tumors of the same histological type. The expression of different common antigens is complementary, so tumor cells that lack a particular antigen may express other types of tumor antigens [28]. Thus, a viable strategy for circumventing the antigenic heterogeneity of tumors is to construct polyvalent vaccines containing a broad range of widely expressed immunogenic tumor antigens.

4. Potentiating vaccine immunogenicity

Tumor associated antigens are weak immunogens. As an example, antibody and/or cellular response to melanoma in patients with this cancer are the exception rather than the rule [29–31]. Methods must be developed to boost the activity of those antigens that are capable of inducing immune responses in humans.

Three general strategies are available to augment the immunogenicity of antigens: 1) Modifying their physical or biochemical properties or presenting them in multimeric units that are more immunogenic. 2) Administering the antigen together with an adjuvant. 3) Using immunomodulators to increase the ability of the host to respond to the antigen and to drive the responses that are induced in a desired direction. These approaches are not mutually exclusive. It is likely that combinations will have an additive or synergistic effect.

Examples of antigen modifications that have been attempted to augment the immunogenicity of melanoma antigens includes physical aggregation of the antigens, neuraminidase treatment [8], and infecting the melanoma cells with live nonpathogenic viruses [5, 7, 11]. Melanoma antigens have been coated unto liposomes or incorporated into vaccinia virus to enable their presentation in multimeric units. No comperative studies are available to judge the relative efficacy of these procedures.

The classic approach to augmenting the immunogenicity of vaccines is to use an adjuvant. Unfortunately, adjuvants that are safe for humans, such as alum, are not very potent, while potent adjuvants such as BCG or Freund's complete adjuvant can cause severe skin reactions. A number of newer adjuvants have been developed which, it is claimed, retain the potency of mycobacterial adjuvants without toxicity. Full clinical evaluation of these adjuvants in humans is still pending, so their effectiveness is not known.

There is increasing interest in using immunomodulators such as cyclophosphamide, interleukin-2, and other lymphokines alone or in combination in order to more vigorously drive forward immune responses that are stimulated by vaccines or to inactivate suppressor machanisms that may depress the response. The most extensively studied immunomodular is cyclophosphamide (see below). The effect of these agents on the immunogenicity of vaccines in humans is still unclear. However, one concern is that many immunomodulators are toxic in their own right, and/or require repeated administration in a hospital or physician's office, which negates a major attractiveness of tumor vaccines – their safety and ease of use.

5. Stimulation of inappropriate immune responses

Immune responses to tumors are complex both in

their variety and in their effect. While some responses may damage tumor cells and hinder tumor growth, others may protect these cells and enhance their growth [32]. Thus a critical aspect of vaccine development is the identification of beneficial immune responses, as evidenced by a correlation between the presence of such a response and a favorable clinical outcome. As described below, we have found such a correlation between the stimulation of a delayed type hypersensitivity response to vaccine immunization and a delay in tumor recurrence.

An aspect of this problem that has been the object of particular attention is suppressor cell function, which may depress immune responses to active immunization to tumor antigens. Several strategies are available to minimize suppressor cell activity. Immunization with low doses of antigens [33] and intradermal administration of antigens [33] both reduce suppressor cell function. Cytotoxic drugs such as cyclophosphamide administered 2 to 4 days prior to antigen challenge may selectively inactivate suppressor cells and, in animals, can enhance the immunogenicity of vaccines. Studies in patients with advanced melanoma suggest the same may be true in humans [9]. However, in our study of this agent we did not find that it potentiated the immunogenicity of a melanoma vaccine in patients with early (regional disease) melanoma [34]. As a result, we suspect that the vaccine potentiating activity of cyclophosphamide may be restricted to patients with advanced disease, in whom there may be greater disturbance in suppressor cell function.

6. Tumor load

Animal studies indicate that tumor vaccines are most effective if the tumor load is small. The implications of this finding are twofold: a) Vaccines should be used to treat patients with minimal disease. b) The usual criterion used to evaluate the effectiveness of chemotherapeutic agents, i.e., the ability to cause regression of established disease, should not be used as the critical test of tumor vaccine effectiveness, as it may miss important beneficial effects in preventing tumor recurrence. The regression of established melanoma in vaccine-treated patients with disseminated disease has been reported [36, 10, 9], so vaccine may also be active in patients with advanced disease.

Vaccine design strategy

Our strategy for constructing a vaccine for melanoma was designed to circumvent some of the problems described above. Its major elemets are: a) Preparation from a pool of melanoma cells, selected because they express different patterns of melanoma antigens, in order to create a polyvalent vaccine that can circumvent tumor antigen heterogeneity. b) The use of cultured cells to ensure a continued and reproducible supply of material for vaccine preparation and to permit treatment of patients in whom no tumor tissue is available. c) Adapting the melanoma cells to grow in serum-free medium to exclude these highly immunogenic and undesirable proteins from the vaccine. d) Preparing the vaccine from shed material. e) Further purifying the vaccine to deplete HLA antigens. The elements of this strategy and their advantages in relation to other approaches to vaccine design are discussed below.

1. Selection of cells for vaccine preparation

It is clear from: a) the antigenic heterogeneity of tumors, and b) the lack of information about the nature of tumor antigens that stimulate protective immune responses, that cancer vaccines should contain multiple tumor associated antigens. They will then be more likely to contain immunogenic antigens expressed by the tumor to be treated. For this reason we prepared our vaccine from a pool of four different melanoma cell lines, selected because they expressed different patterns of melanoma associated antigens. As described later, this approach was successful in creating a vaccine that contained multiple melanoma associated antigens (MAAs). Most of the MAAs tested for were present in the vaccine, so it is probable that it contains additional MAAs that were not tested for. At least one of the MAAs present in the vaccine was ex-

pressed by each of 23 metastatic melanomas that we immunophenotyped, so the antigen mix in the vaccine seems appropriate for circumventing the antigenic heterogeneity among melanoma.

An alternate strategy for dealing with tumor antigen heterogeneity is to prepare vaccine from autologous tumor cells obtained from the patient to be treated. There are several problems with this approach. The first is that autologous vaccines do not circumvent the problem of tumor antigen heterogeneity. The antigen profile of different tumor cells in the same person is variable. As a consequence, vaccine prepared from tumor nodule A will not necessarily contain the antigens expressed by tumor nodule B in the same individual. Autologous vaccines are impractical, since an individual vaccine must be prepared for each patient. Finally, perhaps the greatest drawback of autologous vaccines is that they require a fair amount of tumor tissue for their preparation, so they cannot be used in the patients with minimal disease who are the best candidates for vaccine immunotherapy, in patients whom no fresh tumor tissue is available, or prophylactically to prevent disease.

Another consideration in selecting tumor cells for vaccine preparation is whether they should be freshly obtained from surgically excised tumor tissue or maintained in tissue culture. Fresh tumor tissue is more likely to express antigens relevant for immunotheraphy. However, its use is impractical for the reason discussed with autologous antigens – the need to make individual vaccine for each patient and the inability to treat patients with no available tumor tissue. Cultured cells, on the other hand, provide an economical and reproducible source of material for vaccine production and enable the preparation of generic vaccines that can be used to treat all patients with the same histological type of tumor, as well as patients who have no tumor tissue available.

The cells used to prepare our vaccine have been adapted to grow in serum-free medium in order to avoid contaminating the vaccine with this undesirable material. FCS proteins are highly immunogenic, and appear to be responsible for many of the immune responses induced by human tumor vaccines prepared from cultured cells [37–39]. This approach seems to have been successful, since no FCS protein can be detected in our vaccine [36]. It should be noted that because FCS protein adheres tightly to cells, simply washing tumor cells or incubating them briefly in serum-free medium will not get rid of the FCS protein.

3. Collection of tumor antigens for vaccine production

A key step in our method of vaccine preparation is to obtain the antigens used to construct the vaccine from surface material shed by the melanoma cells. It is based on the principle that tumor cells rapidly release into the culture medium a broad representation of cell surface components, including tumor antigen [40, 41]. Shed material has several advantages as a source of tumor antigen for vaccine construction. It is easily collected, in a manner that can be scaled up for commercial production. The tumor antigens are partially purified, as they are separated from the bulk of unrelated cellular components that are in the cytoplasm and are poorly released in the short collection periods used. As a result the shed material is enriched in surface antigens [41], which are most relevant for immunotherapy. The antigens can be repeatedly harvested from the same cells, reducing culture requirements.

There are other methods for obtaining tumor antigens for vaccine preparation. Most involve the use of whole tumor cells. The cells are either used intact [12, 9], or after they have been broken up mechanically with detergents or other treatments, or lysed with non-pathogenic viruses such as vaccinia [5, 11, 7], Newcastle disease [5], or VSV [38]. The bulk of the material in such vaccines is derived from the cytoplasm, rather than from the surface of the cells, and is thus irrelevant to vaccine activity. The presence of irrelevant antigens in the vaccine may diminish immunogenic activity through antigenic competition. Vaccines prepared from whole cells are contaminated with nuclear material that creates increasing safety concerns as the vaccine is used to treat less advanced disease. Vaccines prepared from viral oncolysates present the additional

safety concern that they contain live viral particles, although this is counterbalanced by the possibility that the viral components increase the immunogenicity of the tumor antigens.

Ideally, vaccines should be constructed from pure tumor antigens. Unfortunately, this approach seems premature, since the tumor antigens that should be used for this purpose – antigens that can induce strong tumor protective immune responses in humans – are not known.

4. Vaccine purification

As it is prognosed from shed antigens, the vaccine is already fairly purified as it is separated from the bulk of cytoplasmic and nuclear cellular material which is poorly shed. The vaccine is put through a further simple purification procedure intended to deplete HLA antigens, which involves detergent treatment and ultracentrifugation. Much of the material shed by melanoma cells is in fragments or vesicles. These can be broken up with detergent to release tumor antigens in a soluble form that remains in the supernatant after ultracentrifugation [41]. However, as others have found and we confirmed [41], ultracentrifugation still sediments detergent treated transplantation antigens, providing a simple way of separating HLA from tumor antigens.

Properties of vaccine

The vaccine contains multiple melanoma-associated antigens (MAA), including the 240+ kD proteoglycan antigen (described by S. Ferrone and Dr. R. Reisfeld), the p97 kD MAA (described by Dr. K. Hellstrom), the 26, 29, 95, 116 kD antigen (described by Dr. H. Koprowsky), and 75, 95, 120, 140, 150, and 240 kD MAAs defined by polyclonal antisera raised in our laboratory. A number of the antigens in the vaccine have been shown to be immunogenic in humans (see below). It is free of fetal calf serum proteins [36]. The vaccine can be made reproducibly, as three batches prepared on different occasions contained the same pattern of melanoma associated antigens [36]. For use, the vaccine is bound to alum as an adjuvant.

Steps in evaluating a tumor vaccine

The clinical evaluation of cancer vaccines involves three major steps: a) Evaluating safety in humans. b) Evaluating biological activity or immunogenicity – can the vaccine stimulate antibody/cellular immune responses to the tumor, and if so, how often. c) Evaluating clinical effectiveness – can the vaccine slow the progression of the tumor.

Safety

Our vaccine has been administered to over 200 patients. There has been no toxicity other than transient urticaria at the injection site [43]. A small asymptomatic granuloma, caused by the alum, remains at the injection site for several months. There has been no skin ulceration.

Other cancer vaccines have been administered to several thousand patients. No toxicity due to the intrinsic properties of the vaccines themselves has been reported. The enhancement of tumor growth resulting from the stimulation of inappropriate immune responses, or the induction of autosensitization, are theoretical risks. No clear evidence of these phenomena has been reported.

The side effects of vaccine immunotherapy have been caused, not by the vaccines themselves, but by the adjuvants (such as BCG, Freund's complete, or incomplete adjuvant) given to enhance the activity of the vaccine. These can cause persistent ulceration at the site of injection, and fever. It can be expected that side effects will also result from some of the immunodulators now being considered to boost vaccine immunogenicity. As the use of tumor vaccines is extended to patients with milder disease or to prevention of disease in healthy but high-risk individuals, the concern for safety will increase. Specific areas of concern include the presence of nuclear material or live viral particles in some vaccine formulations.

In summary, tumor vaccines appear to be rela-

tively safe, and are associated with fewer side effects than conventional chemotherapy or other forms of immunotherapy.

Immunogenic properties of vaccine

This is an important attribute of a vaccine's activity, as it is unlikely that a vaccine will be clinically effective if it cannot induce or augment immune responses to the tumor being treated.

In addition, the type and frequency of immune response induced by a vaccine may provide early clues as to its clinical effectiveness. For this reason, an important area of investigation is the correlation between parameters of the immune response to vaccine immunization and clinical outcome.

Evaluation of a vaccine's immunogenic potential involves measuring the antibody and/or cellular response (preferably both) it is able to induce in humans, and determining whether these responses are directed to tumor as opposed to unrelated antigens. In the case of our vaccine, we found that antibody (measured by protein A-sepharose immuno recipitation) and cellular (measured by DTH response to skin tests to vaccine) immune responses to melanoma were induced or augmented by immunization in 24% and 51%, respectively, of the first 55 patients treated [43].

It is important to realize, when studying the immunogenic potential of vaccines, that the incidence of immune responses detected will depend heavily on the sensitivity of the assay used. As an example, we detected vaccine-induced antibody response to melanoma in 61% of 26 sequential patients using a recently improved immunoprecipitation SDS-PAGE analysis assay [26, 27] but in only 11% of these patients using our former assay.

The antibodies induced by our vaccine are directed to one or more cell surface antigens with approximate molecular weights of 200+, 150, 110, 75, or 38 kDs [26]. The most immunogenic antigens are the 200+ kD and the 110 kD molecules [27]. Both are melanoma-associated, as they are preferentially expressed on melanoma cells. Both antigens were expressed on four of five human melanomas, but on only two of 12 control cell lines. These antigens are not related to HLA molecules based on their molecular weight, nor to FCS proteins, as antigen binding is not blocked by an excess of cold FCS.

The DTH response to the vaccine was carefully analyzed, as it correlates with an improved clinical outcome. DTH responses were induced in a variable (18% to 54%) proportion of patients, depending on the immunization procedure used. The most immunogenic regimen involved using alum as an adjuvant [35].

The DTH response appears to be selectively directed to melanoma. None of the first 17 patients with a DTH response to the vaccine reacted to skin tests to an equal amount of a control vaccine prepared from allogeneic lymphocytes pooled from five different normal donors [43]. Since pooled lymphocytes are a rich source of a broad spectrum of class I and II MHC antigens, it is unlikely the DTH response induced by the melanoma vaccine is directed to MHC antigens. Only 1 of the 24 patients with a strong DTH response to the vaccine reacted to skin tests with concentrated complete culture medium used to grow melanoma cells, making it unlikely that the DHT response is induced by a culture medium contaminant.

The vaccine also stimulates a T cell response which can be measured *in vitro* and which is selectively directed, at least in part, to melanoma. *In vitro* cytotoxicity was measured sequentially before and after vaccine immunization in 18 pts by Dr. Aliza Adler using a direct 4 hr ^{51}Cr release assay. Periphereal blood lymphocytes (PBL) were the effector cells, and a panel of the four melanoma cell lines used for vaccine preparation (M14, M20, HM54, SK-Mel-28) and four control cell lines (melanoma [SK-Mel-23], colon carcinoma [SK-CO1], K562, Daudi) were the targets. Enhanced cytolytic activity to melanoma following vaccine immunization was detected with a frequency that depended on the melanoma cells used as targets. The most sensitive target was M20 melanoma. Vaccine treatment enhanced cytolytic activity to these cells in all patients studied. In two patients, this response was directed selectively to melanoma, i.e., cytolytic activity was strongly augmented to M20 and weakly to M14 and SK-Mel-28 melanoma cells, but was

unchanged against control cells. In the other patients PBL killed both melanoma and some non-melanoma cells, suggesting that immunization stimulates both melanoma selective and non-selective cytolytic cellular responses.

The vaccine can stimulate a cellular immune response to a patient's own tumor. Dense (> 15 cells/high power field) infiltrates of tumor infiltrating lymphocytes (TIL) were more frequent in subcutaneous metastases removed from vaccine-immunized patients (10 of 11 patients, 91%) than in similar metastases removed from non-immunized patients (9 of 22, 41%, p = 0.02) [42].

Clinical effectiveness of melanoma vaccine immunization

Randomized, concurrently controlled, clinical trials have not yet been carried out with any melanoma vaccine. Consequently, the clinical effectiveness of melanoma vaccines in slowing the progression of melanoma is still uncertain, but there are a number of promising preliminary observations. In advanced disease, we [36] and others [9, 10] have observed occasional regression of established disease. However, as the course of melanoma is erratic it is unclear to what extent these favorable responses are the result of vaccine treatment.

As an alternate approach to examining the impact of vaccine treatment on the progression of melanoma, we have correlated the ability of the vaccine to induce an immune response to clinical outcome. The analysis was conducted in 99 sequential patients with post-surgical stage II (regional disease) melanoma. The DTH response to the vaccine was measured prior to and following the fourth vaccine immunization. There was a relationship between the magnitude of vaccine-induced increase in DTH response and prolongation in disease-free (DF) survival. Median DF survival was 4 years longer in the patients with a strong increase in DTH response to the vaccine than in non-responders (> 65 vs 12 months, respectively). By Cox proportional hazard analysis the relationship between the strength of the DTH response and the increase in DF survival was significant (P = 0.01), and could not be accounted for by differences in desease severity (thickness or level of primary lesion, age, sex, number of involved regional nodes, nodes clinically positive at presentation) or immune status (as evaluated by responses to recall antigens or by sensitization to DNCB). There is also a correlation between the ability of an immunization procedure to stimulate DTH response and delay in tumor recurrence [35].

These results indicate that the DTH response to vaccine immunization correlates with the clinical outcome. This finding suggests that vaccine immunization may be effective in delaying tumor progression in patients in whom the vacccine can stimulate a DTH response.

Prevention of cancer with vaccines

An exciting potential application of cancer vaccines is the prevention of cancer. This is a reasonable possibility, as it can be done in animals (as described earlier). Hopefully, this can also be accomplished in humans. The prevention of cancer may in fact prove easier to accomplish than the treatment of established disease. In mice, it is easier to prevent melanoma with vaccines than to slow its progression once it is present [24]. Similarly, vaccines for infectious diseases are more effective in preventing than in treating disease. If the melanoma vaccines currently being developed prove to be safe to use and effective in patients with established melanoma, it will be reasonable to examine whether they can prevent melanoma in patients at high risk of developing this disease. As a result of the considerable progress that has been made in identifying risk factors for melanoma, such individuals can be identified with a fair degree of precision; this includes persons with large congenital nevi or with familial dysplastic nevi syndrome and a family history of melanoma.

Conclusions

Cancer vaccines are a conceptually attractive meth-

od for treating and possibly preventing cancer, for the following reasons:

1. They can prevent cancer in animals.
2. They are relatively safe to use, and have fewer side effects than conventional chemotherapy and other forms of immunotherapy.
3. They are biologically active, and can stimulate immune responses to cancer in some patients, including responses directed to the patient's own tumor.
4. Preliminary studies suggest they may be able to slow the progression of some cancers in humans.
5. Because cancer vaccines are relatively safe, they are particularly attractive as adjuvant therapy in early disease, a stage in the evolution of most cancers for which there are no effective therapeutic options after surgery.
6. Cancer vaccines are unique in their potential application to preventing cancer.

Key unanswered questions

Researchers should be considering the following topics:

1. The identity of tumor antigens which are immunogenic in humans and which should be used for vaccine construction.
2. The appropriate mix of immunogenic tumor antigens required to construct polyvalent vaccines that can circumvent the antigenic heterogeneity of tumors.
3. The development of appropriate methods for boosting the immunogenicity of vaccines.
4. The identification of vaccine-stimulated host effector mechanisms that are important in protective immunity.
5. The demonstration that vaccines are clinically effective by conducting randomized, concurrently controlled clinical trials.

Acknowledgements

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ANTIBODY RESPONSE TO IMMUNIZATION WITH GANGLIOSIDE GD3 AND GD3 CONGENERS (LACTONES, AMIDE AND GANGLIOSIDOL) IN PATIENTS WITH MALIGNANT MELANOMA

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GD3 is the ganglioside most abundantly expressed on the cell surface of human melanoma, and treatment with a murine MAb recognizing GD3 has induced major responses in a small proportion of patients with melanoma. We have therefore attempted to induce production of GD3 antibodies in melanoma patients by active immunization. We found, however, that vaccination with GD3-expressing melanoma cells or purified GD3 does not result in antibody production. We describe here attempts to overcome the poor immunogenicity of GD3 in patients with melanoma by chemical modification. GD3 lactones, GD3 amide and GD3 gangliosidol were synthesized, and the humoral immune response to these derivatives was analyzed. Immunization of melanoma patients with these GD3 derivatives resulted in production of IgM antibodies and, in the case of GD3 amide, also of IgG antibodies. The antibodies to the GD3 derivatives did not cross-react with GD3. This is in contrast to observations in the mouse, where GD3 lactone I induced antibodies that showed cross-reactivity with GD3. Thus, the human immune response was specifically directed toward the modified epitope, rather than to the native structure.

In studies of the humoral immune response to ganglioside vaccines in patients with malignant melanoma, it has been shown that GM2 is consistently immunogenic, that GD2 elicits an antibody response only rarely and that GD3 does not induce production of antibody (Tai *et al.*, 1985; Livingston *et al.*, 1987*b*). GD3, however, is of particular interest for vaccine construction because it is the most abundantly expressed ganglioside of human melanomas (Portoukalian *et al.*, 1976; Pukel *et al.*, 1982), and because treatment with an MAb recognizing GD3 has induced major responses in a small proportion of patients with melanoma (Houghton *et al.*, 1985; Dippold *et al.*, 1985). An approach that has been adopted to enhance the poor immunogenicity of antigens is the construction of chemically [illegible] tion with the native molecule. With regard to gangliosides, Nores *et al.* (1987) have reported that the poor immunogenicity of GM3 in mice can be overcome by immunization with GM3-lactones. In addition, we have shown that GD3 lactones, amide and gangliosidol are immunogenic in mice, and that GD3 lactone I induces production of antibodies that cross-react with GD3 (Ritter *et al.*, 1990*a*). We report here that these GD3 derivatives were also immunogenic in patients with malignant melanoma. In contrast to the situation in mice, however, the antibodies elicited by GD3 amide, GD3 lactones and GD3 gangliosidol in humans were specific for the respective immunogens, and showed only weak reactivity with unmodified GD3 in ELISA, no reactivity with GD3 in dot-blot immune stains or ITLC, and no reactivity with human melanoma cells expressing GD3.

MATERIAL AND METHODS

Gangliosides

GM3, GM2, GM1, GD1b and GD3 were provided by Fidia (Abano Terme, Italy). GD2 was prepared from GD1b by treatment with bovine testis β-galactosidase (Distler and Jourdian, 1978). Gangliosides of human melanoma cell lines SK-MEL-19, SK-MEL-21 and SK-MEL-28 were prepared without saponification or peracetylation by published procedures (Momoi and Wiegandt, 1980; Ritter *et al.*, 1987).

Ganglioside derivatives

GD3 derivatives were prepared as described by Ritter *et al.* (1990*a*). Briefly, GD3 lactones were prepared by treating calf brain GD3 with glacial acetic acid (Ando *et al.*, 1989). Lactones were separated according to charge by DEAE-Sephadex A-25 chromatography, eluting lactone II in chloroform/methanol/water 30:60:8 v/v and lactone I in 0.05 M NH_4Ac in methanol (Iwamori and Nagai, 1978). GD3 amide was obtained by aminolysis of GD3 lactone II (Sonnino *et al.*, 1983), followed by treatment with 0.05 M NaOH in methanol for 60 min at 37°C. GD3 gangliosidols were obtained by reduction of GD3 lactone II with sodium borohydride (Nores *et al.*, 1987). All derivatives were further purified by Sephadex LH-20 chromatography using chloroform/methanol 1:2 v/v as eluent. TLC characteristics and structures of GD3 and GD3 derivatives are shown in Figure 1.

Monoclonal antibodies and enzymes

Rabbit anti-human IgM, rabbit anti-human IgG and goat anti-mouse IgG conjugated with alkaline phosphatase were obtained from Zymed (San Francisco, CA); goat anti-human IgM and goat anti-human IgG conjugated with peroxidase were obtained from Tago (Burlingame, CA). Murine antibodies against human IgG subclasses MCOO2, MCOO3, MCOO4, MCOO6, and MCOO7 were obtained from The Binding Site (Birmingham, UK) and JDC-1, JDC-10, AC3-AA1, C3-8-80, and C27-15 from Southern Biotechnology (Birmingham, AL).

Patients

Patients with melanoma metastases restricted to regional skin and lymph nodes were considered eligible if the skin metastases and regional lymph nodes had been resected within the previous 4 months and if they were free of detectable melanoma. None of the patients had received prior chemotherapy or radiation therapy. Examination of patients, including neurological examination, was performed at 6-weekly intervals. Chest X-rays, liver function tests and urine analysis were

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Abbreviations: The designations GM3, GM2, GM1, GD3, GD2, GD1a and GD1b are used in accordance with the abbreviated ganglioside nomenclature proposed by Svennerholm (1963). GSL, glycosphingolipid; MAb, monoclonal antibody; TLC, thin-layer chromatography; ITLC, immune thin-layer chromatography; HPLC, high-performance liquid chromatography; IA, immune adherence; PA, protein A; ELISA, enzyme-linked immunosorbent assay.

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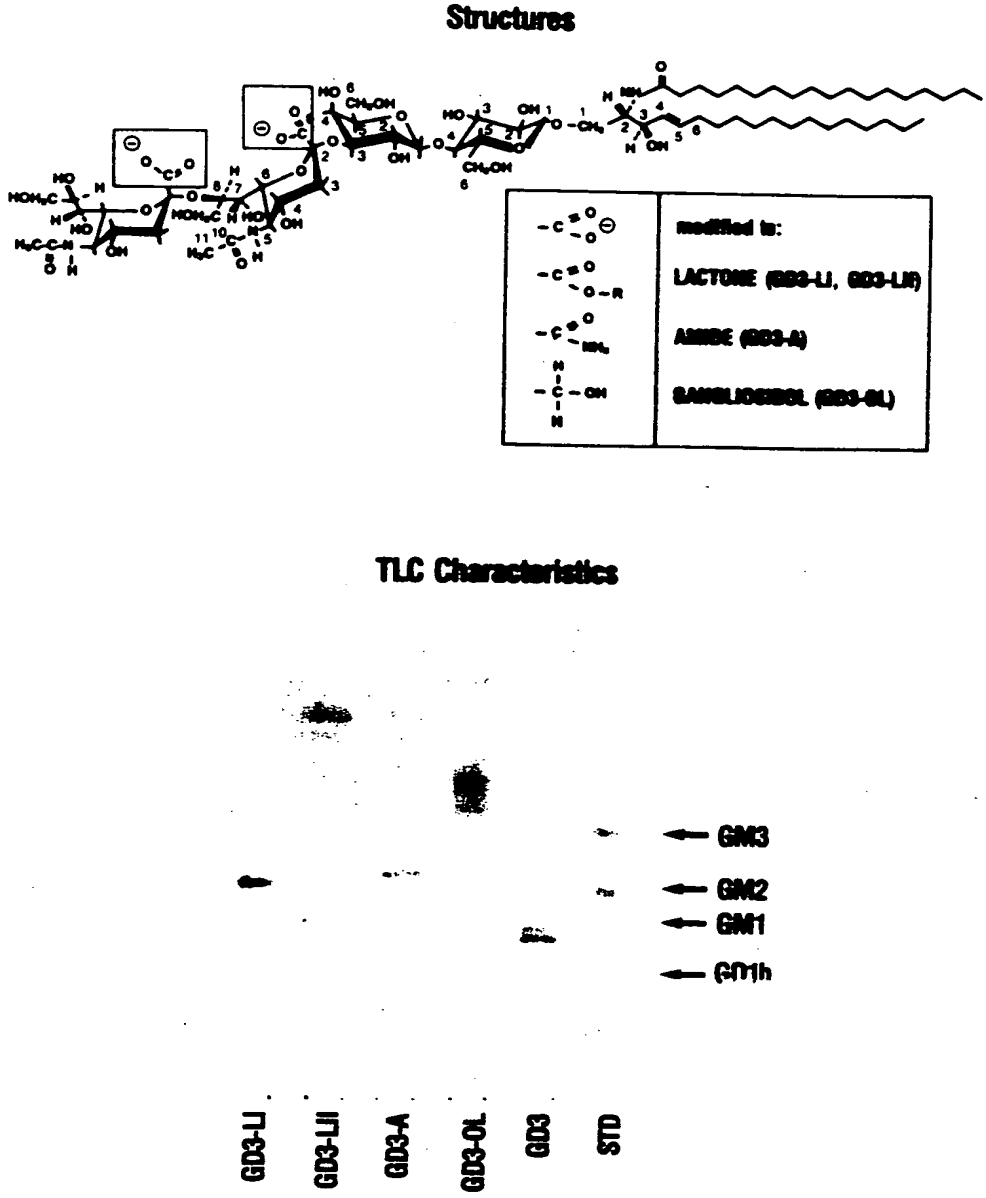

FIGURE 1 – Structures and thin-layer chromatography characteristics of ganglioside derivatives used in this study. HPTLC on silica gel plate; running solvent chloroform/methanol/0.02% aqueous $CaCl_2$ 60:35:8 v/v; staining reagent: orcinol/H_2SO_4.

obtained at 3-monthly intervals. Blood for serological studies was obtained at 2-weekly intervals.

BCG-ganglioside vaccines

Ten million viable units of BCG (Tice strain, University of Illinois, Chicago, IL), or 3×10^6 units in the case of patients showing strong reactions to BCG, were suspended in distilled water and added to tubes containing dried, purified gangliosides. The suspension was lyophilized and the residue suspended in phosphate-buffered saline shortly before vaccine administration. Following this procedure, all gangliosides were attached to BCG, presumably by hydrophobic interactions, as previously reported for the ganglioside GM2 (Livingston *et al.*, 1987*a*). Vaccines containing 100 μg of an individual ganglioside per injection were injected intradermally into extremities with intact lymphatic drainage at 2-weekly intervals for a total of 3 vaccinations. In addition, a booster vaccine injection was administered 2–3 months after completion of the initial series of vaccinations unless there was evidence of recurrence.

Cyclophosphamide administration

Cyclophosphamide (Cytoxan, Mead Johnson, Syracuse, NY) 200 mg/m^2 was administered i.v. to all patients 4–6 days prior to the first vaccine injection.

Skin tests for delayed-type hypersensitivity (DTH)

Twenty-five micrograms of ganglioside suspended in 0.05 ml normal saline were injected intradermally. Skin tests for

DTH against recall antigens were performed and interpreted as described by Pinsky *et al.* (1974).

High-performance thin-layer chromatography

TLC analysis was performed on HPTLC silica gel glass plates (Merck, Darmstadt, Germany). Gangliosides and ganglioside derivatives were separated in chloroform/methanol/0.02% aqueous $CaCl_2$ 60:35:8 v/v solvent and visualized by staining with orcinol/H_2SO_4 or resorcinol/HCl.

Immune thin-layer chromatography

Immunostaining of gangliosides with human sera after separation on HPTLC silica gel glass plates was performed as described (Magnani *et al.*, 1980; Ritter *et al.*, 1987). Peroxidase activity was detected with 4-chloro-1-naphthol.

Enzyme-linked immunosorbent assays (ELISA) and dot-blot immune stains

These assays were performed as described (Ritter *et al.*, 1990*b*). Briefly, ELISA were performed with rabbit anti-human IgM or anti-human IgG conjugated with alkaline phosphatase, and readings were obtained at 414 nm. Antibody titer was defined as the highest serum dilution yielding an OD of over 0.190. Dot-blot immune stains were performed on nitrocellulose membrane strips (0.2μg, Schleicher and Schuell, Keene, NH) using a serum dilution of 1:150 and peroxidase-conjugated goat anti-human IgM and goat anti-human IgG antibodies diluted 1:500. Staining intensity was graded as negative, 1+, 2+ or 3+.

IgG subclass determination

IgG subclass determination was performed by ELISA using subclass-specific murine MAbs. The lowest dilution of the secondary antibody that showed no reactivity with pretreatment serum or other negative control sera was used. Goat anti-mouse IgG conjugated with alkaline phosphatase was used as the third antibody at a dilution of 1:200.

Immune adherence and protein A hemadsorption assays

These assays measure rosetting of human RBC (blood group O) on target cells mediated by IgM (IA) or IgG (PA) antibodies. Assays were performed as described (Shiku *et al.*, 1976; Pfreundschuh *et al.*, 1978).

RESULTS

Antibody response to immunization with GD3 or single GD3 derivatives

Four groups of melanoma patients were immunized with GD3, GD3 lactone I, GD3 lactone II or GD3 amide. The antibody response was analyzed by ELISA and dot-blot immune stain (Table I). Before immunization, antibodies against GD3 and GD3 derivatives were detected only rarely by ELISA and at low titer (<1:40). Immunization with GD3 vaccines did not induce an antibody response. By contrast, vaccination with GD3 derivatives resulted in the production of antibodies. Two of 6 patients immunized with GD3 lactone I developed low-titer IgM antibodies and 5 of 6 patients immunized with GD3 lactone II produced medium- or low-titer IgM antibodies specific for the respective immunogen; no IgG antibodies were detected. GD3 amide elicited the strongest immune response. All 4 patients tested developed high-titer IgM antibodies and moderate- or high-titer IgG antibodies against the immunogen as determined by ELISA, dot-blot immune stains and ITLC. Antibodies of the IgM class were detected 2 weeks after the first vaccination with GD3 amide (Fig. 2*a*), and they reached peak titers first at 2 weeks after the third vaccination, and later again 2 weeks after the booster injection. The immune response was short-lived; antibody titers fell to lower levels over the next 6 weeks. IgG antibodies were first detected 2 weeks after the third vaccine injection (Fig. 2*b*), then declined and rose again 2 weeks after the booster vaccination, with a subsequent decrease to low or undetectable levels over a period of 10 weeks. The IgG subclass of the antibodies induced by immunization with GD3 amide was IgG_1 in 2 cases and IgG_1 as well as IgG_3 antibodies in the 2 other patients. No antibodies of the IgG_2 or IgG_4 subclasses were detected (Table II).

Antibody response to immunization with vaccines containing multiple GD3 derivatives

Six patients were immunized in the same way with a mixture of unmodified GD3, GD3 amide, GD3 lactones I and II and GD3 gangliosidol. The antibody response to the mixed vaccine was analyzed by ELISA and dot-blot immune stains (Table III). Prior to immunization no antibodies reactive with any of the vaccine components were detected. In response to vaccination, 4 patients produced medium- to low-titer antibodies reactive with GD3 amide, GD3 lactones and GD3 gangliosi-

TABLE I – ANTIBODY RESPONSE TO IMMUNIZATION WITH GD3 AND GD3 DERIVATIVES AS DETERMINED BY ELISA AND DOT-BLOT IMMUNE STAIN

			Results of serological analysis[1]							
			ELISA[2]				Dot-blot immune stain[3]			
Vaccine	Serum	Number of patients	Antibodies against the immunogen		Antibodies against GD3		Antibodies against the immunogen		Antibodies against GD3	
			IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
GD3	Before vaccine									
	After vaccine	6	0	0	0	0	0	0	0	0
GD3-lactone I	Before vaccine	6	0	0	0	0	0	0	0	0
	After vaccine	4	40(1),20(1),0(3)	0	0	0	2	1	0	0
GD3-lactone II	Before vaccine	6	0	0	20(2),0(4)	0	1	0	0	0
	After vaccine	5	160(1),80(1), 40(1),20(2)	0	40(1),20(1),0(3)	0	1	0	0	0
GD3-amide	Before vaccine	5	20(1),0(4)	0	40(2),0(3)	0	0	0	0	0
	After vaccine	4	640(1),160(3)	320(2),40(2)	40(1),20(2)	20(2),0(2)	4	4	0	0
GD3-amide/GD3[5]	Before vaccine	6	0	0	0	0	0	0	0	0
	After vaccine	4	160(2),80(1),0(1)	0	0	0	3	2	0	0

[1]Sera used in these assays were obtained 2 weeks after the 4th immunization.–[2]Expressed as reciprocal antibody titers (numbers of patients).–[3]Expressed in number of patients showing reactivity > +2 (reactivity was graded −, +, +1, +2, and +3).–[4]Some patients were removed from study due to disease progression after 2 vaccinations or less.–[5]1st and 2nd vaccine GD3-amide, 3rd and 4th vaccine GD3.

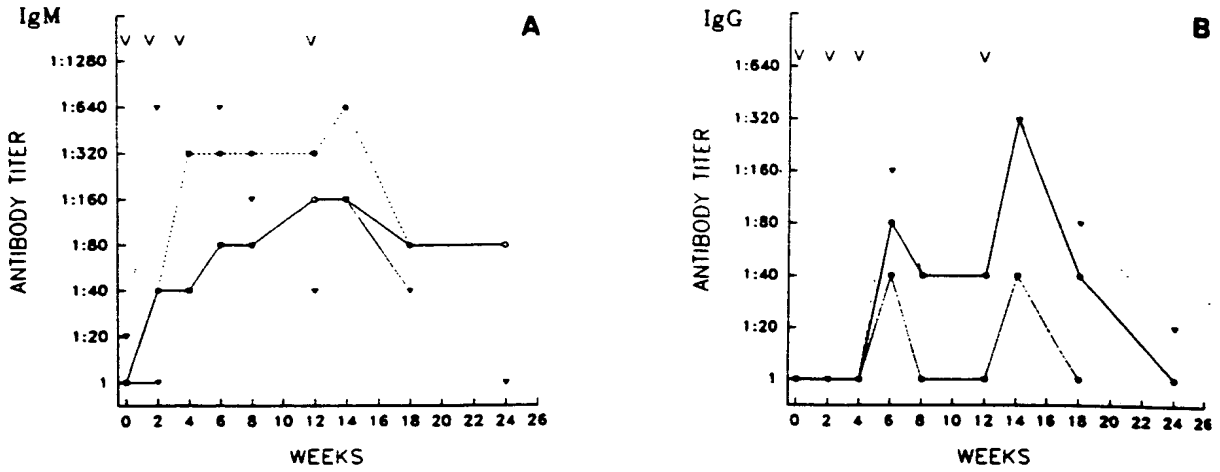

FIGURE 2 – IgM and IgG antibody responses in 4 melanoma patients after immunization with GD3 amide. V indicates vaccine administration.

dol. All antibodies were of the IgM class. Antibodies reactive with GD3 were not detected.

Antibody response to initial immunizations with GD3 amide followed by immunization with unmodified GD3

As GD3 amide was strongly immunogenic in patients with melanoma, we immunized patients with GD3 amide prior to 2 injections with unmodified GD3 to determine whether the immune response to GD3 could be enhanced by a pre-existing immune response to GD3 amide. We found that this was not the case. The results are shown in Table I.

Specificity analysis and cell-surface reactivity of antibodies induced by GD3 derivatives

The specificity of ganglioside antibodies detected in the patients' sera before and after vaccination was further analyzed by dot-blot immune stain on ganglioside standards GM3, GM2, GM1, GD3, GD2, GD1a, and GD1b, and by immune thin-layer chromatography on ganglioside standards and on gangliosides extracted from the 3 melanoma cell lines (SK-[illegible]e by the GD3 derivatives were specific for the respective immunogen and showed no reactivity with other gangliosides tested including GD3 (Fig. 3) and gangliosides extracted from human melanoma cells. In addition, sera from patients immunized with GD3 derivatives were tested in IA and PA assays for cell-surface reactivity with human melanoma cell lines expressing high levels (SK-MEL-19), moderate levels (SK-MEL-28) or undetectable levels (SK-MEL-31) of cell-surface GD3. None of the sera showed reactivity, indicating that these GD3 derivatives are not expressed on the surface of GD3-expressing human melanoma cells, and that the antibodies induced by immunization with GD3 amide, GD3 lactones and GD3 gangliosidol do not cross-react with cell-surface GD3 of human melanoma cells.

TABLE II – SUBCLASS CHARACTERIZATION OF IgG ANTIBODIES INDUCED BY GD3 AMIDE

IgG subclass antibodies		Titer serum of patients			
Specificity	Antibody source	A	B	C	D
IGG	JDC-10	80	80	10	640
	MC002	40	40	0	160
IGG1	JDC-1	40	40	20	80
	MC003	40	20	10	80
IGG2	AC3-AA1	0	0	0	0
	MC004	0	0	0	0
IGG3	C3-8-80	40	0	0	320
	MC006	20	0	0	80
IGG4	C-27-15	0	0	0	0
	MC007	0	0	0	0

Sera used for subclass characterization were obtained 2 weeks after the boost injection; mean value of 2 determinations expressed as reciprocal titers.

Absence of delayed-type hypersensitivity (DTH) to GD3 and GD3 derivatives

Skin tests for DTH to common antigens, PPD, GD3 and GD3 derivatives were performed after the initial series of 3–4 vaccinations. All patients showed reactivity with at least one recall antigen, and all developed strong reactivity to PPD, (as a consequence of BCG in the vaccine) but none showed reactivity to unmodified GD3 or to any of the GD3 derivatives.

DISCUSSION

Of the gangliosides found on the cell surface of human melanomas, GD3 is of particular interest because it is most abundantly expressed. As [illegible] with a murine MAb recognizing GD3 has been shown to induce regression of melanoma metastases in a small proportion of patients (Houghton *et al.*, 1985; Dippold *et al.*, 1985) we attempted to induce production of GD3 antibodies by vaccination with whole cells expressing cell-surface GD3, or with purified GD3. These attempts failed, however, although antibodies to other melanoma gangliosides, GM2 and GD2, could be induced by active immunization (Tai *et al.*, 1985; Livingston *et al.*, 1987*b*, 1989*b*). This experience led us to search for ways in which the poor immunogenicity of GD3 could be improved.

Several reports indicate that chemical modification of gangliosides may augment their immunogenicity. Synthetic ganglioside congeners that have been reported to induce antibody production in laboratory animals more readily than the unmodified parent molecule include GM1 methylester, GM1 gangliosidol and GM1-N-methylamide (Handa and Nakamura, 1984; Nakamura and Handa, 1986), GM3 lactone (Nores *et al.*, 1987), GD3 amide, GD3 gangliosidol, GD3 lactones I and II and O-acetyl GD3 (Ritter *et al.*, 1990*a*, 1990*b*). Furthermore, serological cross-reactivity between ganglioside derivatives and their parent molecules has been reported. Murine MAbs reactive with ganglioside lactones are also reactive with the parent gangliosides (Dohi *et al.*, 1988; Tai *et al.*, 1988; Ando *et al.*, 1989; Bosslet *et al.*, 1989; Ritter *et al.*, 1989). Immunization of mice with GM3 lactone induced antibodies that cross-reacted with unmodified GM3 (Nores *et al.*, 1987), and

TABLE III – ANTIBODY RESPONSE TO IMMUNIZATION WITH A MIXED VACCINE CONTAINING GD3, GD3 AMIDE, GD3 LACTONES AND GD3 GANGLIOSIDOL

Test antigen	Results of serological analysis[1]							
	ELISA[2]				Dot-blot immune stains[3]			
	Before vaccine		After vaccine		Before vaccine		After vaccine	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
GD3	0	0	40(1),0(5)	0	0	0	0	0
GD3 Amide	0	0	640(1),80(1), 40(2),0(1)	0	0	0	4	0
GD3 Lactones	0	0	80(2),40(2),0(2)	0	0	0	0	0
GD3 Gangliosidol	0	0	80(2),40(1), 20(1),0(2)	0	0	0	0	0

[1]Sera used in these assays were obtained 2 weeks after the 4th immunization.–[2]Expressed as reciprocal titers (numbers of patients).–[3]Expressed in number of patients showing reactivity > +2 (reactivity was graded −, +, +1, +2, and +3).

a murine MAb reactive with both GM3 and GM3 lactone has been generated (Dohi *et al.*, 1988). Serum antibodies induced by immunization of mice with GD3 lactone I were shown to react with purified GD3 and GD3-expressing human melanoma cells (Ritter *et al.*, 1990*a*). Although GD3 is clearly a poor immunogen in humans, there is some evidence that it can be recognized by the human immune system. Low-titered GD3-reactive antibodies hav · been described in the serum of some melanoma patients (R. vindranath *et al.*, 1989), and human MAbs reactive with GD3 have been generated (Yamaguchi *et al.*, 1987).

Putting together these observations—increased immunogenicity of synthetic ganglioside congeners in mice, cross-reactivity of congener-induced murine antibodies with their parent gangliosides, and the basic ability of the human immune system to produce antibodies recognizing GD3—it seemed reasonable to synthesize GD3 congeners with the expectation that they might be more immunogenic than GD3 in human melanoma patients, and that the induced antibodies might cross-react with GD3. Immunization with GD3 amide, GD3 gangliosidol and GD3 lactones I and II resulted, in fact, in the production of IgM antibodies, and also IgG antibodies in the case of GD3 amide. This strong immunogenicity of GD3 derivatives is most likely due to the fact that these modified molecules are not normally expressed, and may be, in addition, more resistant to metabolic degradation (Li *et al.*, 1984; Handa [illegible] 1987). The antibody re- sponses were short-lived and antibody titers could not be boosted, indicating induction of a primary response but not memory B-cells or affinity maturation. The patients did not develop delayed-type hypersensitivity to the GD3 derivatives. This pattern of response was identical with that observed in patients after immunization with the ganglioside GM2 (Livingston *et al.*, 1989*b*) and is similar to the T-cell-independent immune response against bacterial or synthetic carbohydrate antigens (Howard, 1987).

The antibodies produced by the patients in response to immunization with GD3 derivatives, including GD3 lactones, were specific for the immunogen and did not significantly cross-react with purified melanoma GD3 or calf brain GD3, or with cell-surface GD3 on human melanoma cells. Furthermore, successful immunization with GD3 derivatives did not condition the patient for a subsequent response to GD3 vaccines. We have made similar observations with GD2 lactone vaccines; they too induce an antibody response to GD2 lactones but not unmodified GD2 in patients with melanoma (data not shown). Because GD3 and GD2 are expressed on a variety of normal human cell types in addition to malignant melanoma (Garin-Chesa *et al.*, 1989), a state of immunologic tolerance to these gangliosides is likely to exist. A less likely possibility is that antibodies to these gangliosides are induced after immunization but rapidly adsorbed by cells normally expressing GD3 or GD2. These alternatives can be further explored by analysis of anti-ganglioside antibody production at the clonal level using EBV-transformation and hybridoma technology in vaccinated and non-vaccinated patients.

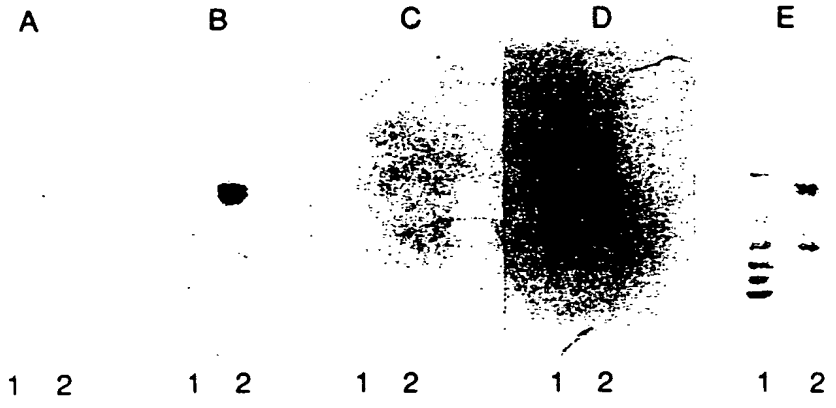

FIGURE 3 – Reactivity of sera from melanoma patients with gangliosides after immunization with GD3 amide *(b)* and GD3 amide followed by GD3 boosts *(d)* as determined by immune thin-layer chromatography. Pre-immune sera from the same patient *(a, c)*. Plate *(a)* counterstained with Resorcinol *(e)*. GM3, GM2, GM1, GD1a, GD1b, and GD2 (lanes 1); GD3 amide and GD3 (lanes 2); HPTLC on silica gel plates; running solvent chloroform/methanol/0.02% aqueous $CaCl_2$ 60:35:8 v/v; serum dilution 1:50; staining peroxidase and 4-chloro-1-naphthol *(a–d)*; additional resorcinol stain *(e)*.

The immunodominance of ganglioside lactone epitopes over other epitopes on the carbohydrate moiety in humans contrasts with the findings in mice, where GM3 lactone and GD3 lactone I are effective in inducing GM3- or GD3-reactive antibodies, respectively. In the case of GD3, the basis for this difference may be that the ganglioside is widely expressed in human but not in murine tissues (Livingston *et al.*, 1989*a*). In the case of GM3, the immunogenicity of GM3-lactone has not been tested in humans, so a direct comparison cannot be made.

Although GD3 derivatives are better B-cell immunogens in man than the naturally occurring ganglioside, they do not appear to elicit T-cell recognition or help. As has been seen with other gangliosides as well as with non-ganglioside carbohydrate antigens, antibody titers were low and short-lived despite repeated immunizations. It appears clear that, in addition to the specific problem of how to make GD3 immunogenic, the general issue of improving the efficacy of presentation of this entire class of antigens needs to be addressed. Approaches that are attractive for exploration include the testing of adjuvants that may substitute for T-cell help (Lowell *et al.*, 1988), and construction of vaccines linking poorly immunogenic carbohydrate epitopes to structures that facilitate T-cell recognition (Tam and Lu, 1989; Snider *et al.*, 1990). The rapid advances that are now being made in the general field of antigen presentation and recognition will no doubt benefit efforts to develop immunogenic ganglioside vaccines.

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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
D,P, X	PROCEEDINGS OF THE 84TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 34, March 1993, page 491 XP002023381 HELLING ET AL: "INCREASED IMMUNOGENICITY OF GM2 CONJUGATED WITH KLH AND USED WITH ADJUVANTS IN PATIENTS WITH MELANOMA" * abstract 2929 * ---	1-20	A61K45/05 A61K31/70 A01N43/08 A61K39/00 A61K39/385
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A	CANCER AND METASTASIS REVIEWS, vol. 9, 1990, pages 81-91, XP000616188 BYSTRYN: "TUMOR VACCINES" * the whole document * -----		

The supplementary search report has been drawn up for the claims attached hereto.

1

Place of search	Date of completion of the search	Examiner
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